

**PULMONARY COLONISATION OF PATIENTS WITH CYSTIC FIBROSIS
BY *PSEUDOMONAS AERUGINOSA***

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ABBREVIATIONS

Abbreviations frequently used and/or novel to this thesis are listed below.

BAC	benzyltrimethylalkyl-ammonium chloride
BAPS	biotinylated alkaline phosphatase-streptavidin
bio-Mab(s)	biotinylated monoclonal antibody
BSA	bovine serum albumin
CF	cystic fibrosis
CM	chemotaxis medium
ELISA	enzyme-linked immunosorbent assay
GalNAc	N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
HRP	horse-radish peroxidase
IATS	International Antigenic Typing Scheme
LPS	lipopolysaccharide
Mab(s)	monoclonal antibody
MCP	methyl-accepting chemotaxis protein
MM	minimal media
<i>Mr</i>	relative molecular weight
Muc	muroid
NA	nutrient agar
NANA	N-acetylneuraminic acid
NC	no chemotaxis
NCIB	National Collection of Industrial Bacteria
NCTC	National Collection of Type Cultures
NIC	nitrocellulose
NT	non-typable
NYB	nutrient yeast broth

OD	optical density
PA	polyagglutinating antigen
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEV	<i>Pseudomonas</i> extract vaccine
PIA	<i>Pseudomonas</i> isolation agar
PVA	<i>Pseudomonas</i> vaccine antigen
R-LPS	rough lipopolysaccharide
SAP	streptavidin alkaline phosphatase
SD	standard deviation of the mean
SDS	sodium dodecylsulphate
S-LPS	smooth lipopolysaccharide
spp	species (plural)
SUR	streptavidin urease
TBS	tris buffered saline
TTBS	Tween tris buffered saline

ABSTRACT

Chronic respiratory colonisation by the adaptable opportunistic pathogen *Pseudomonas aeruginosa* is a major debilitating feature of the inherited disease cystic fibrosis (CF). This thesis considers various aspects of the pathogenesis of *P. aeruginosa* in CF, including the serological response to bacterial colonisation, and possible factors involved in early colonisation.

Anti-*P. aeruginosa* lipopolysaccharide (LPS) antibodies in sera, saliva and sputa from patients with CF were measured by enzyme-linked immunosorbent assay (ELISA) incorporating either a polyvalent *Pseudomonas* smooth LPS extract vaccine, or *P. aeruginosa* core, rough LPS. Elevated levels of anti-LPS IgG antibodies in serum, and IgA antibodies in saliva and sputum were demonstrated in patients chronically colonised by *P. aeruginosa*. Low levels of serum anti-LPS IgG antibodies were detected in some patients intermittently colonised by *P. aeruginosa* but not in non-*P. aeruginosa* colonised patients. Anti-LPS IgA antibodies were detected in some of both intermittently and non-colonised patients. Immunoblot analysis of serum IgG and sputum IgA antibodies to *P. aeruginosa* LPS revealed a response directed towards O-antigenic LPS in the initial stages of pulmonary colonisation with non-mucoid *P. aeruginosa* and a response towards common core LPS during subsequent chronic infection with mucoid *P. aeruginosa*.

Flagellar preparations from *P. aeruginosa* strains were characterised and used in ELISA and immunoblot studies to detect anti-*P. aeruginosa* flagellar antibodies in sera, saliva and sputum. Serum anti-flagellar IgG antibodies and salivary and sputum anti-flagellar IgA antibodies were detected, particularly in those CF patients intermittently or chronically colonised by *P. aeruginosa*. Antibodies to both type-a and -b flagella were detected; in some patients a pronounced antibody response to only one of the flagellar types was evident.

Anti-*P. aeruginosa* LPS monoclonal antibodies (Mabs) were produced for use in a sandwich ELISA for the detection of *P. aeruginosa* in respiratory secretions of patients with CF. LPS defective mutants expressing only common core LPS were used to immunise mice for preparation of Mabs. Antibodies were screened in ELISA and the antigenic component(s) of LPS recognised by the most *P. aeruginosa* cross-reactive Mabs was checked by immunoblotting. Five IgG Mabs were characterised and found to recognise the core component of *P. aeruginosa* LPS. Two of the Mabs were particularly reactive against core LPS from all O-antigenic serotypes of *P. aeruginosa* and were included in the sandwich ELISA for detection of *P. aeruginosa* LPS. A biotin-streptavidin amplification system was used to increase assay sensitivity. The sensitivity of the assay was 0.1 ng/ml *P. aeruginosa* LPS; the assay was able to detect *P. aeruginosa* LPS in the respiratory secretions from patients with CF.

The chemotactic response of *P. aeruginosa* towards various mucin preparations and major amino acid and sugar components of mucin was investigated. Purified CF mucin, chronic bronchitic mucin, normal salivary mucin, mucin glycopeptides as well as porcine mucin acted as chemoattractants for *P. aeruginosa*. The degree of chemotaxis towards amino acids was also strain specific; optimum chemotaxis was observed towards serine, alanine, glycine, proline and threonine. No strain showed chemotaxis to N-acetylneuraminic acid (NANA) but a strain dependent chemotactic response to the sugars L-fucose, D-galactose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine (GlcNAc) was observed.

Transmission electron microscopy with a surfactant monolayer technique demonstrated adhesion of *P. aeruginosa* to porcine gastric mucin and purified CF mucin. A microtitre ELISA incorporating one of the characterised anti-*P. aeruginosa* Mabs was also used to measure adherence of *P. aeruginosa* to mucin. Strain dependent adhesion to various mucin preparations was

observed, particularly to normal salivary mucins and CF mucin glycopeptides. Sugar inhibition studies indicated that GlcNAc and NANA may constitute the binding site in mucin.

The relevance and significance of these studies is discussed.

PUBLICATIONS

- 1 **NELSON, J.W., BARCLAY, G.R. & GOVAN, J.R.W.** (1988) Development of an alternative enzyme-linked immunosorbent assay (ELISA) for the diagnosis of chronic *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. Basic research and clinical aspects of *Pseudomonas aeruginosa* infection: International Symposium, Copenhagen, Denmark. Abstract 41.
- 2 **NELSON, J.W., TREDGETT, M.W. & GOVAN, J.R.W.** (1989) Mucinophilic and chemotactic properties of *Pseudomonas aeruginosa*. 4th European Congress of Clinical Microbiology, Nice. Abstract 863/PP43.
- 3 **NELSON, J.W., BARCLAY, G.R. & GOVAN, J.R.W.** (1990) Diagnosis of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis by ELISA for anti-*Pseudomonas* LPS IgG antibodies. Serodiag. Immuno. Infect. Dis. **4**, 9-16.
- 4 **NELSON, J.W., TREDGETT, M.W., SHEEHAN, J.K., THORNTON, D.J., NOTMAN, D. & GOVAN, J.R.W.** (1990) Mucinophilic and chemotactic properties of *Pseudomonas aeruginosa* in relation to pulmonary colonization in cystic fibrosis. Infect. Immun. **58**, 1489-1495.
- 5 **NELSON, J.W., GOVAN, J.R.W., BARCLAY, G.R., MICKLEM, L.R. & JAMES, K.** (1990) Development of a sandwich ELISA for the early detection of *Pseudomonas aeruginosa* colonization in patients with CF. Pediatr. Pulmonol. Suppl. **5**, Abstract 313.
- 6 **NELSON, J.W., GOVAN, J.R.W., SHEEHAN, J.K. & THORNTON, D.J.** (1990) Adherence and chemotaxis of *Pseudomonas aeruginosa* to tracheobronchial mucins. Pediatr. Pulmonol. Suppl. **5**, Abstract 190.

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DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise indicated in the acknowledgements.

INTRODUCTION

CHAPTER 1

CYSTIC FIBROSIS

1.1 GENERAL FEATURES

Cystic fibrosis (CF), otherwise known as mucoviscidosis or fibrocystic disease of the pancreas, has been recognised as a disease since the late 1930s (Andersen, 1938), and is the most common lethal genetic disorder of the Caucasian population (Wood *et al*, 1976; Davies & Di Sant' Agnese, 1980). CF is an inherited autosomal defect (Merritt *et al*, 1962; Danks *et al*, 1965; Rommens *et al*, 1989) and is thus manifested when two carriers produce a child inheriting the mutant gene from both parents. The incidence of CF in the Caucasian population is approximately 1 in 2,000 live births with a calculated carrier frequency of 1 in 20 (Wood *et al*, 1976; Brunecky, 1982; Boat *et al*, 1989); there are approximately 5,000 CF patients in the UK (Dodge *et al*, 1988). The incidence is less frequent in other racial groups (Romeo *et al*, 1989) although occasionally detected, eg an incidence of 1 in 17,000 live births amongst negroes in Washington D.C. (Kulczycki & Shauf, 1974).

1.2 CLINICAL FEATURES

There is considerable variability in the expression of CF and scarcely any organ system is exempt from involvement (Fick, 1981). CF is characterised by disturbances in mucus secretion and electrolyte transport from exocrine glands and secretory epithelia (Talamo *et al*, 1983; McPherson & Dormer, 1987; McPherson & Goodchild, 1988). CF pancreatic secretions contain precipitates leading to ductal blockage, acinar cell degeneration and extensive fibrosis; the disease was initially called cystic fibrosis of the pancreas, fibrotic cysts being observed in post mortem pancreatic material from CF patients (Andersen, 1938; Talamo *et al*, 1983). In addition to exocrine pancreatic insufficiency and concomitant

digestive problems, perhaps the most important symptom of the disease is the overproduction of abnormally viscid tracheobronchial mucus which results in poor mucociliary clearance of the bronchial airways, and subsequent bacterial colonisation, pulmonary inflammation, production of copious viscid sputum and chronic obstructive pulmonary disease (McPherson & Dormer, 1987; Govan, 1988). The other consistent feature of CF is the elevated levels of sweat electrolytes and this forms the basis of the pilocarpine-stimulated sweat test used in diagnosis of the disease (McPherson & Dormer, 1987).

Compared to the 1940s and 1950s when CF patients seldom survived infancy, the average life expectancy and prognosis for a CF patient has increased considerably. In the UK 50% of patients with CF now survive to the age of 20 years, (Dodge *et al*, 1988). Meconium ileus (gastrointestinal blockage caused by the inspissation of secretions), which used to be a primary cause of infant mortality in patients with CF, is becoming increasingly rare as a cause of death, due to increased awareness and early diagnosis of CF, improved paediatric surgery and neonatal intensive care, as well as improved patient management (Dodge *et al*, 1988). Other developments over the last decade contributing to improved patient prognosis include improvements in dietary supplements, effective physiotherapy, use of increasingly potent antimicrobial agents and introduction of heart lung transplants, (Dinwiddie, 1990; Govan & Glass, 1990). Today, progressive pulmonary disease exacerbated by intermittent debilitating respiratory infections presents the main threat to both quality of life and life expectancy in CF patients (Govan & Glass, 1990).

1.3 THE CYSTIC FIBROSIS GENE

Intensive individual and collaborative studies contributed to the eventual identification of the defective gene in individuals with CF (Rommens *et al*, 1989; Riordan *et al*, 1989). Initially linkage analysis based on a large number of polymorphic DNA markers was used to localise the gene to a region in the middle of the long arm of chromosome 7, between the flanking markers *met*

and *D7S8* (White *et al*, 1985; Beudet *et al*, 1986). Novel gene cloning techniques including chromosome walking and jumping, and complementary DNA hybridisation enabled further refinement of the map and identification of the relatively large CF gene extending over 250 kb of genomic DNA (Rommens *et al*, 1989). Messenger RNA transcripts, 6.5 kb in size constructed from 24 exons, were detected in various tissues of patients with CF including the pancreas, lung, nasal polyps, sweat glands, colon and liver, but not in unaffected tissue eg brain (Riordan *et al*, 1989). Further genetic analysis of the candidate gene showed that 68% of CF patients have a single mutation resulting in a deletion of three nucleotides encoding phenylalanine at position 508 of the proposed protein (Kerem *et al*, 1989). Work is currently underway to determine the nature and extent of the other mutations (Scrambler, 1989).

The benefits of these advances include improved methods for screening of heterozygous carriers and for prenatal diagnosis, as well as a confirmation of CF in doubtful cases (Scrambler, 1989; Knight & Hodson, 1990). A complete description of all the CF mutations will aid towards a full understanding of the pathophysiology of the disease and form a basis for the development of improved therapy (Kerem *et al*, 1989).

1.4 BIOCHEMICAL DEFECT AND PATHOPHYSIOLOGY OF CYSTIC FIBROSIS

The discovery of the CF gene also made it possible to predict a likely structure and function of the protein the gene encodes. Analysis of the sequence of overlapping complementary DNA clones predicted a polypeptide gene product of 1,480 amino acids. The main characteristics of the predicted protein structure are two repeated motifs, each consisting of six membrane spanning domains, and sequences resembling nucleotide (ATP) binding folds (Riordan *et al*, 1989).

Available evidence suggests that the cystic fibrosis transmembrane regulator

(CFTR) is likely to be involved in the transport of substances across the membrane either by acting as an ion channel or regulating ion channel activities. Since the CFTR appears to belong to a family of proteins, many of which function as parts of multicomponent molecular transport systems, the CFTR protein may be involved in other epithelial cell functions including secretory activity (Riordan *et al*, 1989). Although the fundamental biochemical defect in CF remains uncertain, available evidence suggests defective chloride ion transportation in CF airway epithelia and in isolated sweat gland reabsorptive ducts (Knowles *et al*, 1983; Quinton, 1983). The reduction in chloride permeability resulting in a net decrease in the reabsorption of sodium, is a likely explanation for the consistent observation of increased sodium, potassium and chloride content of CF sweat and other secretions, including the mucous secretions seen in CF (McPherson & Dormer, 1987). Recent work suggests that the CF protein is not a chloride ion channel as was first thought (Riordan *et al* 1989). Instead, researchers have proposed that the CFTR transports a regulatory protein which in turn acts on the ion channels to produce the characteristic chloride imbalance (Ringe & Petsko, 1990).

In the future, therapeutic stratagems may involve replacement of the abnormally functioning protein by gene or protein therapy or remedying the defect by drugs (Knight & Hodson, 1990). It is interesting to note available evidence suggesting that transplanted lungs in CF patients do not acquire the electrochemical defect (Alton *et al*, 1987).

A further consequence of the defect seems to be the overproduction of a normal serum protein, the CF antigen (Bullock *et al*, 1982), which is located on chromosome 1 (Van Heyningen *et al*, 1985). The deduced amino acid sequence of the CF antigen revealed extensive similarity to several calcium binding proteins eg calmodulin (Dorin *et al* 1987). One possibility is that the CF antigen is part of a macromolecular signal-transducing complex coupled to the chloride channel with the CF gene product occurring as another component of

the complex (Dorin *et al*, 1987; Goodfellow, 1987).

A number of observations have indicated that cell calcium homeostasis may be disturbed in CF (Case, 1984). Elevated calcium concentrations in CF cells and in CF respiratory, intestinal and salivary secretions have been reported (Case, 1984; Katz *et al*, 1984). The increased anionic character of the mucous glycoproteins in CF may promote increased sequestration and release of calcium (Wood *et al*, 1976). In addition, abnormalities in protein and calcium concentrations in CF secretions (Katz *et al*, 1984) may contribute to observed precipitations which eventually lead to blockage of exocrine gland ducts (McPherson & Dormer, 1987). Calcium might also play a part in decreasing the solubility of glycoproteins and *Pseudomonas aeruginosa* alginate in CF (Di Sant' Agnese & Talamo, 1967; Govan *et al*, 1983).

1.5 PULMONARY INFECTION IN PATIENTS WITH CYSTIC FIBROSIS

Chronic, progressive lung disease exacerbated by intermittent, debilitating respiratory infections is the major determinant of morbidity and mortality in patients with CF (Højby, 1984; Friend, 1986; Govan & Glass, 1990). Histologically, the lungs of infants with CF are normal at birth and comparable with those of non-CF infants (Wood *et al*, 1976; Oppenheimer, 1981). However, soon after birth hypertrophy of bronchial glands, goblet cell hyperplasia, metaplasia of the bronchiolar epithelium followed by mucopurulent plugging of peripheral airways all contribute to the early pulmonary pathology of CF. The sticky, mucopurulent, stagnant secretions gradually fill the bronchioles and bronchi; bacterial colonisation follows, stimulating further mucus secretion and bronchitis and bronchiolitis frequently result. A vicious cycle of obstruction, chronic infection and further respiratory tissue damage develops, and exacerbations resulting from viral or bacterial infection may lead to pneumonia. Bronchiolectasis, bronchiectasis, peribronchial fibrosis and airway obstruction result in the progressive loss of pulmonary function. Once bronchiectasis has developed there are frequent exacerbations and eventually respiratory failure,

cor pulmonale and death (Wood *et al*, 1976; Fick, 1981; Fick & Hata, 1989). Pulmonary disease and remodelling in CF is usually diffuse but more pronounced in the upper lobe segments of the lungs, especially the right (Wood *et al*, 1976; Fick & Hata, 1989).

"The microbiology of lung disease in patients with CF is a sub speciality unto itself" (Stutman & Marks, 1987). Microbial infections tend to be localised in the lung with emphasis on the major and minor airways rather than the alveoli, and localised infections at non-pulmonary sites or systemic infections are rare (Govan & Glass, 1990). Although most infections associated with the chronic, progressive lung disease and episodes of acute exacerbation of CF are due to bacteria, infections with viruses, chlamydia, mycoplasma and fungi also contribute to a state of pulmonary deterioration (May *et al*, 1972; Friend, 1986; Govan & Glass, 1990). The bacteria most commonly isolated from the sputum in CF are *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa* (May *et al*, 1972; Mearns, 1980; Høiby, 1982; Friend, 1986; Govan & Glass, 1990). Susceptibility of individual CF patients to particular bacterial pathogens is usually but not invariably age-related, the sequence usually beginning with *Staph. aureus* colonisation in infancy, followed by *H. influenzae* (non-capsulated, non-group B) in the early years with the incidence of *P. aeruginosa* becoming more frequent with increasing age (May *et al*, 1972; Mearns, 1980; Govan & Glass, 1990).

In the early 1980s reports from several North American clinics cited an increasing isolation (up to 20%) of *Pseudomonas cepacia* from CF patients indicating that this species, possessing intrinsic resistance to many antimicrobials, could present a significant problem (Isles *et al*, 1984). However, it is possible that cross-infection and/or iatrogenic colonisation was responsible for the reported increases in *P. cepacia* rather than the fact that the organism was genuinely emerging as a major new pathogen in CF (Friend, 1986; Govan & Glass, 1990). The current incidence of *P. cepacia* in CF patients in the UK is

stable and currently less than 6% (Govan & Glass, 1990).

Staph. aureus exhibits a range of virulence factors and prior to the discovery of antistaphylococcal agents many patients with CF succumbed to the acute infectious complications caused by *Staph. aureus* (Marks, 1990). In the first bacteriological study of CF lung disease Di Sant' Agnese & Anderson (1946) noted the destructive potential of *Staph. aureus*. Their report based on 14 post-mortem examinations showed *Staph. aureus* from 12 patients, and single isolations of *H. influenzae* and *P. aeruginosa* (*Bacillus pyocyaneus*) each in conjunction with a predominant growth of *Staph. aureus*. Significantly all but two of the cases involved patients less than one year of age and the single isolate of *P. aeruginosa* was from one of the oldest patients who had died aged three years. Since the introduction of effective anti-staphylococcal drugs in the 1950s the incidence of *Staph. aureus* infection in CF has declined whilst that of *P. aeruginosa* has increased, although *Staph. aureus* remains an important cause of respiratory damage and distress in CF patients (Wood *et al*, 1976; Mearns, 1980; Friend, 1986; Govan & Glass, 1990).

The incidence of *P. aeruginosa* in CF patients typically increases with age and most CF centres have experienced an inexorable rise in isolations over the last four decades to a level of 70-80% in adolescent patients (Mearns, 1980; Govan & Glass, 1990). There are, however, significant differences in the isolation rates reported in individual centres (Friend, 1986; Bauernfeind *et al*, 1987; Govan *et al*, 1987; Govan & Glass, 1990) ranging from 30% in Edinburgh and Melbourne clinics to 70-90% in the London and Munich clinics. The reasons for the generally increased incidence of *P. aeruginosa* in CF patients, the variation in the isolation rates for *P. aeruginosa* in different centres and the influence of antistaphylococcal therapy on the subsequent colonisation by *P. aeruginosa* are uncertain and controversial.

Since *P. aeruginosa* has emerged as the predominant pathogen in the antibiotic era it has been suggested that anti-staphylococcal therapy has caused the increased incidence of *P. aeruginosa* in CF patients (Huang *et al*, 1961; Burns & May, 1968; Kulczycki *et al*, 1978; Kulczycki *et al*, 1988; Govan & Glass, 1990). In addition as an initial pathogen, *Staph. aureus*, may cause damage to the lung rendering it susceptible to subsequent colonisation by *P. aeruginosa* (Burns & May, 1968; Kilbourn, 1978). Alternatively, the switch from *Staph. aureus* to *P. aeruginosa* and the increased incidence of *P. aeruginosa* could be due to a change in patient population as a result of increased life expectancy (Mearns *et al*, 1972). In the preantibiotic era the majority of children probably did not live long enough for us to be certain that the switch would not have occurred with age, perhaps as antistaphylococcal immunity developed (Ramphal & Vishwanath, 1987). There have been cases reported where *P. aeruginosa* was the first documented pathogen to be isolated from patients who have not previously received antistaphylococcal treatment (Stern *et al*, 1977).

Prior lung damage may be a predisposing factor to *P. aeruginosa* colonisation. However, Høiby (1982) observed that 50% of the CF patients attending the Danish clinic were colonised with *P. aeruginosa* and that some, but not all had impaired lung function at the time when *P. aeruginosa* was first isolated.

Since the frequency of *P. aeruginosa* in CF increases with age, clinics with high adolescent populations would tend to yield a higher incidence than paediatric clinics (Govan *et al*, 1987). In reality the factors underlying the incidence of *P. aeruginosa* in any given CF clinic are likely to be multifactorial, reflecting both age distribution of the CF population and the antibiotic policies employed (Govan *et al*, 1987; Govan & Glass, 1990).

Lung infections in CF patients caused by *Staph. aureus* and *H. influenzae* can be effectively treated with antibiotics (Høiby *et al*, 1982; Pier, 1985; Jensen *et al*, 1989). In contrast, *P. aeruginosa*, despite significant increases in the

potency of antipseudomonal antibiotics, is notoriously intractable to antibiotic therapy, and once established, is seldom, if ever, eradicated (Høiby *et al*, 1982; Govan *et al*, 1987).

An unusual feature of *P. aeruginosa* colonisation in CF is the very high frequency of mucoid, alginate producing variants of *P. aeruginosa* which are particularly associated with progressive chronic respiratory infection in CF patients (Henry *et al*, 1982; Høiby, 1984; Pier 1985; Govan & Glass, 1990). Although mucoid *P. aeruginosa* were first described by Sonnenschein in 1927, the association of mucoid isolates with CF patients was first reported by Iacocca *et al* (1963) and by Doggett and colleagues (Doggett *et al*, 1964; Doggett *et al*, 1966; Doggett, 1969). In CF patients primary asymptomatic colonisation is typically with non-mucoid *P. aeruginosa* strains, from which mucoid variants later emerge (Doggett *et al*, 1966; Govan, 1990).

Despite the isolation of *P. aeruginosa* from a wide range of environmental niches and various tissue sites in infected hosts, strains of *P. aeruginosa* do not typically produce mucoid colonies. In contrast, mucoid *P. aeruginosa* are isolated in up to 90% of CF patients colonised with *Pseudomonas* and can be isolated in up to 40% of *Pseudomonas*-positive sputa from non-CF patients with chronic obstructive lung disease and in up to 10% of patients with chronic urinary tract infections (Govan, 1988; McAvoy *et al*, 1989; Govan, 1990).

Respiratory colonisation by *P. aeruginosa*, especially by variants exhibiting a mucoid phenotype, is the major cause of morbidity and mortality in CF and is widely accepted as the major microbial challenge in CF lung disease (Pier, 1985; Jensen *et al*, 1989; Govan & Glass, 1990). The isolation of mucoid *P. aeruginosa* is almost diagnostic of CF in adolescents with chronic pulmonary disease (Reynolds *et al*, 1976) and the emergence of the mucoid phenotype following initial asymptomatic colonisation with non-mucoid *P. aeruginosa* has acquired ominous status as a "harbinger of death" (Pier, 1985).

CHAPTER 2

PSEUDOMONAS AERUGINOSA

2.1 GENERAL CHARACTERISTICS

The genus *Pseudomonas* comprises more than 200 species, many of which are ubiquitously found in nature particularly in soil and water (Palleroni, 1984). Members of the genus *Pseudomonas* are defined as Gram-negative, straight or slightly curved unicellular rods, measuring 0.5-1 μm in diameter by 1.5-5.0 μm in length, and motile by one or several polar flagella (Palleroni, 1975; Palleroni, 1984). Metabolism is strictly aerobic with oxygen as the terminal electron acceptor, although in some cases nitrate can be used as an alternative electron acceptor allowing growth to occur anaerobically (Palleroni, 1975; Palleroni, 1984). All members of the genus are chemoorganotrophs and many exhibit great catabolic versatility capable of attacking a large variety of organic compounds (Palleroni, 1975).

Present taxonomic classification of *Pseudomonas* species is based on both ribosomal RNA and DNA homology studies, five groupings being defined on the basis of rRNA homology (Palleroni *et al*, 1973; Palleroni, 1975; Palleroni, 1984). The rRNA homology group I contains the fluorescent species characterised by the production of water-soluble pigments (pyoverdins) and in some species phenazine pigments (Palleroni, 1984). The type species and best defined member of the group is *P. aeruginosa*. Most *P. aeruginosa* strains can be identified on the basis of the characteristic grapelike odour of aminoacetophenone, colonial morphology and production of pyocyanin, a blue, water soluble non-fluorescent phenazine pigment. Indeed the name of the species refers to its most striking and definitive characteristic, a colour resembling copper rust or verdigris, ie green, due to the production of pyocyanin and the yellow-green

fluorescent pigment pyoverdine. Individual colonies of *P. aeruginosa* can occur as five distinct types ranging from dwarf colonies to a large mucoid type (Phillips, 1969); the commonest colonial type is large, low convex with an irregular surface and an edge that is translucent in comparison with the pigmented centre. The optimum growth temperature is 37°C, a characteristic which differentiates *P. aeruginosa* from other fluorescent pseudomonads (Palleroni, 1975). Most strains are motile and typically possess one polar flagellum per cell. The organism, obligately aerobic except in media containing nitrate, is physiologically versatile, having both simple nutritional requirements and the ability to metabolize a variety of organic substances (Palleroni, 1984). In addition *P. aeruginosa* is renowned for its innate resistance to a wide range of anti-microbial agents.

2.2 HABITAT

Given the physiological versatility and adaptability of *P. aeruginosa* it is not surprising that the organism is able to adapt and thrive in a spectrum of ecological niches (Rhame, 1980). *P. aeruginosa*, essentially an aquatic organism, is found in almost any environment where moisture is present and is widely distributed in soil, waterways and sewage (Hoadley, 1977). It is commonly found in sinks, swimming pools and jacuzzis (Price & Ahearn, 1988) and is particularly prevalent in niches in hospital environments including infusion fluids, ophthalmic solutions, in-use dilutions of disinfectants, distilled water preparations, soaps, handcreams, respiratory ventilators and nebulizing humidifiers (Favero *et al*, 1971; Favero *et al*, 1975; Neu, 1983; Morrison & Wenzel, 1984). The organism can also be isolated from many plants, animals and insects and may be found as a commensal in the intestinal and respiratory tracts and on moist skin surfaces of a small proportion of healthy human subjects. Stoodley & Thoma (1970) reported that 8% of normal humans carry *P. aeruginosa* as part of their normal gut flora, whilst Rosenthal & Tager (1975) found that only 2% of normal individuals carried *P. aeruginosa* as part of their normal pharyngeal flora. Higher carriage rates of the organism can be found in

hospitalised patients, particularly those being treated in an intensive care unit, because of the greater exposure to reservoirs of the organism and the selective influence of antibiotic therapy (Stoodley & Thoma, 1970; Neu, 1983). The frequency of intestinal carriage in hospital patients may be as high as 75% (Neu, 1983) and the oropharyngeal colonisation by *P. aeruginosa* and other Gram-negatives also increases with severity of illness (Johanson *et al*, 1972).

2.3 CLINICAL INFECTIONS

Although *P. aeruginosa* is usually harmless towards most healthy, uninjured individuals, the organism is an opportunistic pathogen *par-excellence* and responsible for a diverse range of opportunistic infections in patients compromised by injury, underlying disease, chemotherapy or surgical procedure (Cross *et al*, 1983; Sherertz & Sarubbi, 1983). Indeed the organism can infect almost any external site or organ in the body (Young & Armstrong, 1972). Infection often occurs in hospitalised patients after prior instrumentation or manipulative procedures such as urethral catheterization, tracheostomies, lumbar punctures and intravenous infusions of medications and fluids. Patients debilitated by disease or by age and those undergoing treatments with immunosuppressive agents, corticosteroids, antibiotics and radiation are also particularly susceptible to *P. aeruginosa* infection.

Hospitalised patients may acquire the organism from common environmental sources by contact with human or inanimate vectors or through airborne transmission. Nosocomial *P. aeruginosa* infections are common and varied (Geddes, 1980; Cross *et al*, 1983; Neu, 1983; Sherertz & Sarubbi, 1983), and include infections of burns, eyes, ulcers and bedsores, urinary tract infections, acute pneumonias and bacteraemia.

Certain occupational and recreational factors may also contribute to *P. aeruginosa* infection of healthy individuals: serious eye infections may be caused by contaminated contact lenses or eye drops, particularly to the injured

eye; painful otitis externa in deep sea divers working in the high humidity and poor sanitation is associated with saturation diving (Alcock, 1977); and the skin condition folliculitis or 'jaccuzi rash' is associated with users of the warm, aerated aqueous environment of the whirlpool or jaccuzi (Vogt *et al*, 1982; Ratman *et al*, 1986).

2.4 RESPIRATORY INFECTIONS INVOLVING *P. AERUGINOSA*

Although *P. aeruginosa* is not a pathogen of the normal bronchial lining it is associated with two major forms of respiratory tract infection: acute pneumonia and chronic pulmonary disease. Primary or acute *P. aeruginosa* pneumonia is one of the most common of the nosocomial infections caused by the organism (Sherertz & Sarubbi, 1983). Factors that may predispose to the development of acute pneumonia include tracheal intubation, immunocompromisation, underlying chronic lung disease, thoracic surgery, prolonged treatment in an intensive care unit and age greater than 70 years (Johanson *et al*, 1972; Reynolds & Fick, 1980; Celis *et al*, 1988). Involvement of *P. aeruginosa* in chronic pulmonary disease is most commonly associated with patients with CF although it may cause exacerbations in patients with non-CF chronic obstructive airways diseases including chronic bronchitis and bronchiectasis (Doggett & Harrison, 1969; Fick, 1981; Rivera & Nicotra, 1982; Fick & Hata, 1989).

CHAPTER 3

THE *PSEUDOMONAS AERUGINOSA* CELL ENVELOPE

The cell envelope of *P. aeruginosa*, and Gram-negative bacteria in general, is complex in structure, conferring shape and rigidity on the bacterial cell and functioning as a barrier through which a bacterium interacts with its environment (Costerton *et al*, 1974, Brown & Williams, 1985).

The Gram-negative cell envelope (Figure 1) is composed of three layers: a) an outer membrane, b) the periplasmic space and c) the inner or cytoplasmic membrane. In addition the surface of many Gram-negative bacteria may be covered by a polysaccharide layer forming capsules or slimes (Sutherland, 1977; Sutherland, 1985). The term glycocalyx describes all types of polysaccharide material outwith, but adherent to the cell envelope (Costerton *et al*, 1981). The alginate exopolysaccharide produced by mucoid *P. aeruginosa* is one such glycocalyx.

3.1 STRUCTURE AND COMPOSITION OF THE OUTER MEMBRANE

The outer membrane of Gram-negative bacteria is an asymmetric bilayer structure made up of phospholipids, proteins and lipopolysaccharide (LPS) (Costerton *et al*, 1974; Nikaido & Nakae, 1979; Lugtenberg & Van Alphen, 1983; Hammond *et al*, 1984; Nikaido & Vaara, 1985). The outer leaflet of the bilayer is largely made up of LPS, the lipid component linking hydrophobically to the phospholipids making up the inner leaflet, whilst the polysaccharide component extends outwards from the cell surface. The outer membrane also possesses protein components including lipoproteins, major and minor outer membrane proteins and the tubular protein polymers of the flagella and fimbriae.

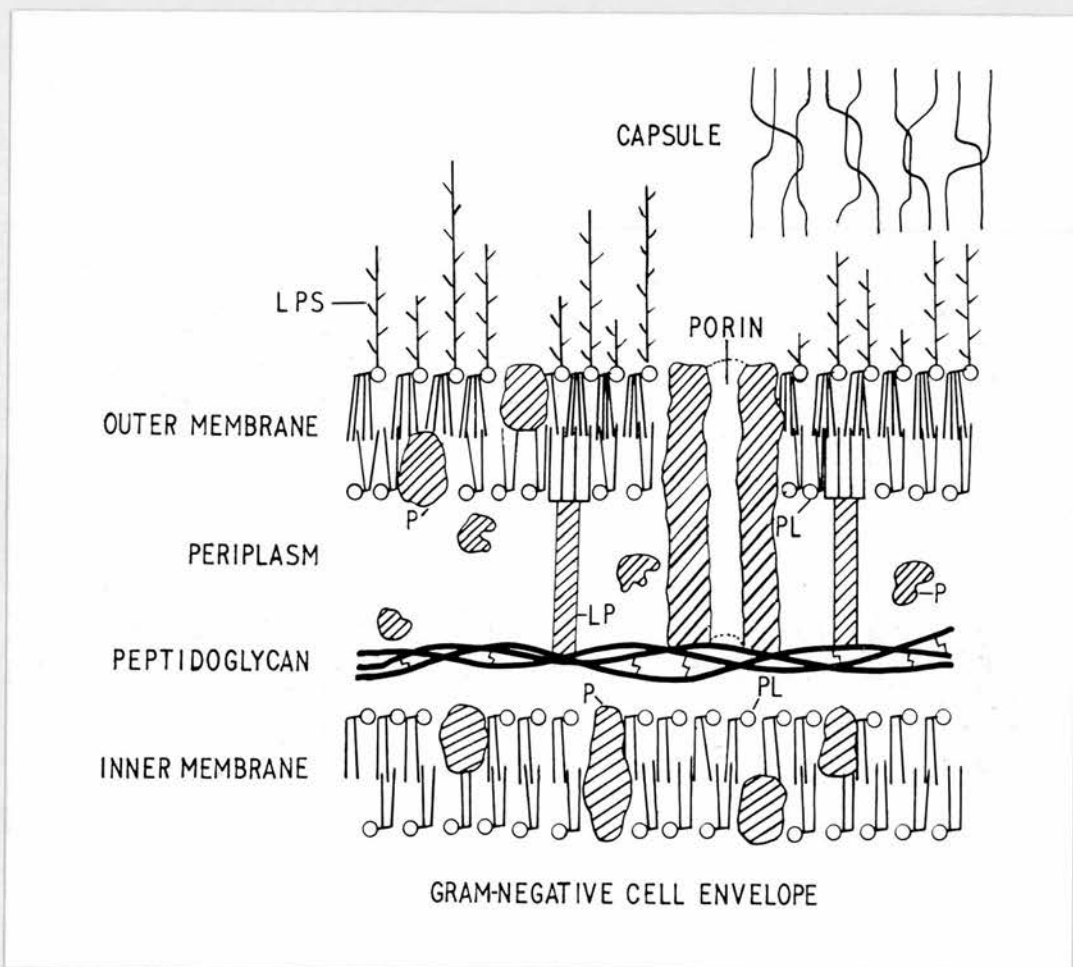


Figure 1. The cell envelope of a Gram-negative bacterium.
 LP = lipoprotein; LPS = lipopolysaccharide; P = protein; PL = phospholipid. (From Hancock & Poxton, 1988).

3.2 LIPOPOLYSACCHARIDE

Lipopolysaccharides are a heterogeneous collection of molecules unique to the outer leaflet of the outer membrane of Gram-negative bacteria, sharing the same general composition but showing heterogeneity in size (Muhlradt & Golecki, 1975; Day & Marceau-Day, 1982). The LPS of *P. aeruginosa* is similar in general structure to that of the enterobacteria in that it is an amphipathic, tripartite macromolecule (Pitt, 1989). Integrated into the continuum of proteins and phospholipids of the outer membrane is the hydrophobic lipid A region of the LPS, consisting of fatty acids linked to glucosamine. Extending outwards from lipid A is the common core region consisting of a short carbohydrate chain. In smooth strains a longer, polymeric, carbohydrate side chain, known as the O-specific side chain, is attached to and extends outwards from the core oligosaccharide (Wilkinson, 1977; Wicken & Knox, 1980; Westphal *et al*, 1983; Hammond *et al*, 1984). The composition and structure of the side chain are variable but the lipid A and core regions are relatively conserved. Analysis and fractionation of the LPS from *P. aeruginosa* indicate that they share many features of typical enterobacterial LPS as well as possessing several distinctive features (Wilkinson, 1983).

Lipid A

The Lipid A moiety of LPS from different species of Gram-negative bacteria has a structure based on a phosphorylated β 1-6 glucosamine disaccharide backbone to which six or seven saturated fatty acids are attached by ester and amide bonds (Westphal *et al*, 1983; Nikaido & Vaara, 1985). Lipid A is unique among bacterial fatty acids in containing hydroxy fatty acids (Hammond *et al*, 1984). The lipid A of *P. aeruginosa* is similar to that of the enterobacteria but differs in fatty acid composition and the unusual high degree of phosphorylation (Wilkinson, 1983). Typically the fatty acid 3-hydroxytetradecanoic acid is absent from the lipid A of *P. aeruginosa* whilst the fatty acids dodecanoic, hexadecanoic, 3-hydroxydecanoic, 2-hydroxydodecanoic and 3-hydroxydode-

canoic acid are present (Wilkinson & Galbraith, 1975; Wilkinson, 1983). In addition minor amounts of palmitate, palmitoleate, stearate and oleate may be found (Pier *et al*, 1981). The hydroxy fatty acids may be amide or ester linked to glucosamine: in *P. aeruginosa*, the amino moieties of the hexosamines are substituted with 3-hydroxydodecanoate residues whilst 3-hydroxydecanoic, dodecanoic and 2-hydroxydodecanoic acids are ester linked to the backbone or to the hydroxy groups of the other fatty acids (Kropinski *et al*, 1985). LPS from *P. aeruginosa* appears to be a less potent endotoxin than the LPS from other Gram-negative bacteria (Sadoff, 1974; Kropinski *et al*, 1985) and this may be due to the absence of 3-hydroxytetradecanoic acid.

Core oligosaccharides

P. aeruginosa LPS has a core structure which appears to differ significantly from other described LPSs (Day & Marceau-Day, 1982). Reported observations indicate heterogeneity in the core region (Wilkinson & Galbraith, 1975) and that the core composition of the LPS varies with growth conditions (Day & Marceau-Day, 1982).

The core oligosaccharide consists of inner and outer domains. The inner core region contains heptose and the unique sugar 3-deoxy-D-*manno*-2-octulosonate or *keto-deoxy octanoic acid* (KDO) which links the core polysaccharide to lipid A via an acid labile bond (Wicken & Knox, 1980). The heptose constituent contains equal amounts of L-glycero-D-*manno*-heptose and D-glycero-D-*manno*-heptose (Hammond *et al*, 1984). In contrast to enterobacterial LPS which contain about four phosphate residues in the inner core region and an additional two or three in the lipid A, LPSs from *P. aeruginosa* may contain ten or more residues per molecule (Kropinski *et al*, 1979; Wilkinson, 1983; Kropinski *et al*, 1985). Inorganic orthophosphate is the major product of mild acid hydrolysis, but small amounts of inorganic pyrophosphate and of ethanolamine ortho-, pyro-, and triphosphates can also be detected (Wilkinson & Galbraith, 1975; Wilkinson, 1983). The outer core of *P. aeruginosa* typically

contains D-glucose, L-rhamnose, D-galactosamine and L-alanine (Wilkinson, 1983).

The composition of the core oligosaccharides from a number of *P. aeruginosa* strains have been analysed and various structures proposed (Drewry *et al*, 1975; Wilkinson & Galbraith, 1975; Rowe & Meadow, 1983). The major components and their molar proportions in the core polysaccharide of *P. aeruginosa* strain NCTC 1999 (National Collection of Type Cultures) were glucose (3-4), rhamnose (1), heptose (2), KDO (1), galactosamine (1), alanine (1-1.5) and phosphorus (6-7), (Drewry *et al*, 1975). However, the core oligosaccharide obtained and analysed from *P. aeruginosa* PACIR differed in the glucose rhamnose ratio (nearer to 3:1 than 4:1), the type of bonding and the number of heptose residues from the core structure proposed for NCTC 1999 (Rowe & Meadow, 1983).

There are a number of controversial areas concerning the core structure of LPS in *P. aeruginosa* (Kropinski *et al*, 1985). Evidence that L-alanine occurs mainly in N-alanylgalactosamine residues needs to be reassessed in light of isolation of a core defective LPS containing alanine but not galactosamine (Jarrell & Kropinski, 1981). In addition the number of phosphate residues and their distribution also requires reassessment (Kropinski *et al*, 1985). Allowing for analytical imprecision, microheterogeneity in the core region, the presence of smooth-rough products, and the effects of cultural conditions, it is likely that a family of related core oligosaccharides exist for *P. aeruginosa* (Wilkinson, 1983).

O-side chains

The O-side chain component of LPS consists of repeating oligosaccharide subunits. The side chains of most *P. aeruginosa* strains contain unbranched tri- or tetrasaccharide repeating units rich in N-acetylated aminosugars including hexosamine, fucosamine and quinovosamine and occasionally bacillosamine

(Wilkinson, 1983; Kropinski *et al*, 1985; Knirel *et al*, 1988). Neutral sugars including glucose, rhamnose, xylose and ribose are not commonly found in *P. aeruginosa* O-polysaccharides and only rhamnose is encountered frequently (Knirel *et al*, 1988). Acidic monosaccharides are also found and two unique bicyclic hexosamines have been isolated from certain O-side chains: an acetylated imidazoline derivative of 2,3-diaminomannuronic acid and a 6,3-lactam of 2,3-diaminoglucuronic acid. Other chemical characteristics of *P. aeruginosa* O-side chain LPS include the presence of uronic acid residues in certain serotypes and the lack of cis-vicanyl hydroxyls in the side chains (Kropinski *et al*, 1985).

Heterogeneity in the size of the *P. aeruginosa* LPS molecule have been reported (Chester & Meadow, 1975; Rivera *et al*, 1988) and attributed to the variations in the number of repeating units in the O-antigen. The number of repeating units can show considerable variation under different physiological conditions (Chester & Meadow, 1975; McGroarty & Rivera, 1990) and even within a culture of one strain (Rivera *et al*, 1988). Such variation confers great variability on the molecular make up of the bacterial cell surface and there is evidence to suggest that a strain may produce several different LPSs containing a common polysaccharide (Chester & Meadow, 1975; Koval & Meadow, 1977; Rivera *et al*, 1988; Lam *et al*, 1989; Rivera & McGroarty, 1989). Indeed analysis of the LPS from PAO1 revealed two antigenically and chemically distinct molecules termed A- and B-band LPS (Rivera *et al*, 1988). B-band LPS is generally composed of LPS with long-chain O-antigen and is the LPS responsible for the O-specificity of the organism, while A-band is composed of LPS molecules consisting of only short-chain polysaccharides (Rivera *et al*, 1988) and may be a common LPS antigen in all *P. aeruginosa* strains (Lam *et al*, 1989; Rivera & McGroarty, 1989).

Compared to the enterobacteria the overall capping frequency of *P. aeruginosa* LPS (ie the percentage of core LPS constituents covered with O-antigen) is low, leaving a greater percentage of core determinants exposed at the cell

surface (Kropinski *et al*, 1985). The percentage of smooth type molecules in *P. aeruginosa* LPS have been reported at 0.2 - 13.7% (Hancock *et al*, 1983), 5-11% (Wilkinson, 1983) and 8% (Rivera, 1988). The unusually high amount of phosphorus in the core of *P. aeruginosa* LPS may be due to the high proportion of core molecules lacking O-side chains (Pitt, 1989).

The great variability in the chemical composition of the O-antigen subunit forms the basis of immunological typing of *P. aeruginosa* strains (Chester *et al*, 1973; Bergan, 1975). The polysaccharides of different O-serogroups differ from one another in monosaccharide composition and structure of their repeating units (Knirel *et al*, 1988). Liu *et al* (1983) proposed a scheme comprising 17 O-types, the International Antigenic Typing Scheme (IATS) which accounted for the types in various previously described schemes (Pitt, 1988) including the original 12 O-types of Habs (1957) and five antigens from other schemata. Three new major somatic antigens of *P. aeruginosa* have recently been described and added to the other 17 IATS groups (Liu & Wang, 1990). Frequencies of individual serotypes in clinical specimens varies with country of investigation and source of the isolate (Pitt, 1988). Serotypes O:6 and O:11 together account for about 40% of *P. aeruginosa* strains isolated worldwide (Pitt, 1989). However, in chronic infections such as CF, bronchiectasis and urinary tract infections, *P. aeruginosa* variants which are defective in their ability to synthesize O-specific side chains often arise from parent O-typable forms (Pitt, 1989). These variants are frequently polyagglutinable (ie agglutinated by more than one antiserum towards unrelated O-antigens) and non-typable as a result of loss of O-side chains (Hancock *et al* 1983; Fomsgaard *et al*, 1988a). Unlike rough forms of enterobacteria, serologically rough strains of *P. aeruginosa* are seldom colonially rough in appearance (Pitt, 1988). In addition to the loss of O-serotype reactions, *P. aeruginosa* isolates from CF patients with chronic bronchopulmonary infections also expressed a new somatic antigen called the polyagglutinating antigen on their surface Penketh *et al* (1983). This antigen may be related to the short-chain polysaccharide A-band LPS which has been

suggested as the major LPS antigen in non-typable clinical isolates, as well as a common antigen among other *P. aeruginosa* strains (Lam *et al*, 1989).

Biological properties of *P. aeruginosa* LPS

Numerous biological activities have been attributed to *P. aeruginosa* LPS including pyrogenic, mitogenic and anti-tumour activity (Homma, 1971; Kropinski *et al*, 1985; Pitt, 1989). Injection of *P. aeruginosa* LPS into mice elicits the classical symptoms of endotoxic shock (Homma, 1971) whilst antibody towards LPS is highly protective both in man and experimental animal models (Pollack & Young, 1979; Cryz *et al*, 1983). LPS plays a major role in the virulence of the organism conferring resistance to the bactericidal action of complement in normal human serum and protection of the bacterial cell against opsonization and phagocytosis (Engels *et al*, 1985).

3.3 PROTEINS OF THE OUTER MEMBRANE

There are three major classes of protein found in the outer membrane including lipoproteins, major outer membrane proteins (defined as components present in sufficient quantity to be readily detected by simple staining of polyacrylamide gels) and minor proteins which are inducible or derepressible and may, under the appropriate environmental conditions, reach levels comparable with those of the major proteins, eg iron regulated membrane proteins.

A number of proteins have been identified and characterised in the outer membrane of *P. aeruginosa* (Hancock & Nikaido, 1978; Mizuno & Kageyama, 1978; Hancock & Carey, 1979). Many of the major outer membrane proteins are pore proteins, porins, which form relatively non-specific, water filled pores spanning the outer membrane for the passive entry of low molecular weight, hydrophilic solutes present at relatively high concentrations (around 10^{-6}M) (Nikaido & Nakae, 1979; Nikaido & Vaara, 1985; Nakae, 1986). The porins also serve as receptors for bacteriocins and bacteriophages (Lugtenberg & van Alphen, 1983). Entry of essential solutes which are too large to pass through the

porins and which are present at very low concentrations (down to 10^{-20}M) proceeds after binding of substrate or a substrate chelator complex to outer membrane receptor proteins (Brass, 1986). It has been reported that a number of the porins in *P. aeruginosa* are small in diameter (Yoshihara & Nakae, 1989) and together with the tightly packed LPS (as a result of divalent cation binding to the many phosphate residues), makes the outer membrane impermeable to both hydrophobic and hydrophilic antibiotics (Nikaido & Hancock, 1986).

3.4 FLAGELLA

The single polar flagellum of *P. aeruginosa* is a major taxonomic characteristic of the organism (Ansorg, 1978; Palleroni, 1984). Flagella are protein, filamentous cell surface appendages responsible for the motility and chemotaxis of many bacteria (Doetsch & Sjöblad, 1980). Each flagellum is composed of three parts:- (1) the basal body, (2) the hook and (3) the filament.

The basal body is the most morphologically and functionally complex region of the bacterial flagellum containing at least ten distinct polypeptides (Doetsch & Sjöblad, 1980; Hammond *et al*, 1984). It serves to anchor the flagellum to the cell surface and provides the driving force for flagellar rotation. In Gram-negative bacteria the basal body consists of a rod about 27 nm long bearing two pairs of discs. The diameter of the discs is about 22 nm, whilst that of the rod is 7-10 nm. The rings have been named (DePhamphilis & Adler, 1971a & b) and are located as follows:- embedded in the cytoplasmic membrane is the M (membrane) ring, and this is closely associated with a second disc, the S (supramembrane) ring lying between the cytoplasmic membrane and the peptidoglycan layer. It is thought that the M-ring serves as a rotor, and the S-ring as a stator during flagellar rotation (Berg, 1974; Hammond *et al*, 1984, Macnab & Aizawa, 1984). The second pair of discs, the P (peptidoglycan) and L (lipopolysaccharide) rings are tightly associated with the outer membrane. Since P and L-rings are not present in Gram-positive bacteria it has been suggested that they may serve as a bearing permitting rotation of the rod in the

relatively mobile outer membrane (Berg, 1974; Hammond *et al*, 1984).

The hook structure serves as a universal joint between the basal body and the filament. It is generally short, about 70-90 nm long, slightly curved, with a diameter slightly greater than the filament, and consists principally of a single polypeptide subunit (Doetsch & Sjoblad, 1980).

The filament, the distal section of the flagellum, is approximately 20 nm in diameter and 10-20 nm long. This thin, helical propeller is made up of a single protein called flagellin (Doetsch & Sjoblad, 1980). All flagellin antigen preparations from *P. aeruginosa* consist of flagellin molecules with a common N-terminal amino acid sequence and they all lack the amino acids proline, cysteine and histidine (Rotering & Dorner, 1989). It has been reported that the *rpoN* gene product, an alternative sigma factor of *P. aeruginosa*, is required for expression of a set of diverse genes, including the flagellin gene (Totten *et al*, 1990). Flagella from *P. aeruginosa* can be categorised into two major groups designated type-a and -b, based on agglutination (Lanyi, 1970) and immunofluorescence assays (Ansorg, 1978), and the molecular weight of the flagellar antigens (Allison *et al*, 1985). Type-a flagella belong to a heterologous group which can be further subdivided into five partial antigens a_0 , a_1 , a_2 , a_3 , a_4 , (Ansorg, 1978) with *Mrs* ranging from 45,000 to 52,000 (Allison *et al*, 1985). These are found in 16 possible combinations in *P. aeruginosa* flagella, the partial antigen a_0 being present in all a-types with usually one or more of the additional flagellin subtypes (Ansorg, 1978). The b-types comprise a homologous group having a *Mr* of 53,000 (Allison *et al*, 1985). Based on this typing scheme Ansorg (1978) could ascribe 17 possible flagella serotypes to 95% of clinical isolates of *P. aeruginosa* with about 60% of the isolates having b- type flagella. Molecular weight analysis by electrophoretic separation (Allison *et al*, 1985), and amino acid analysis (Rotering & Dorner, 1989) of flagella with the serotype a_0 , failed to discriminate them from flagella of serotype b. The presence of the b or a_0 epitope on all 17 flagella serotypes

described by Ansorg (1978) as well as common N-terminal amino acid sequences, indicate that immunological cross-reactions exist between *P. aeruginosa* flagella of different serotypes (Anderson & Montie, 1987; Rotering & Dorner, 1989).

Chemotaxis

P. aeruginosa swim by the rotation of the polar flagellum enabling the organism to seek out nutrients and find a suitable environment in which to grow. Many bacteria including *P. aeruginosa* are able to respond to their environment by a process known as chemotaxis which includes the ability to respond and swim towards attractants and away from noxious repellents (Adler, 1987). Chemotaxis may be defined as the biased migration of cells in response to a chemical gradient (Lauffenburger *et al*, 1987). Positive chemotaxis describes movement towards a chemical gradient whereas negative chemotaxis denotes movement away from a chemical gradient (Chet & Mitchell, 1976).

Flagellate bacteria use the proton-motive force across the cytoplasmic membrane to drive the rotation of the semi-rigid helical flagella (Berg *et al*, 1982). The bacterial movement generated by the flagellar apparatus can be followed by the aid of a tracking microscope, and is characterised by sequences of steps called runs, during which speed and direction are fairly constant, separated by tumbles in which the cells stop and change direction (Berg, 1971). Runs are relatively long lasting for a duration of a few seconds, while tumbles last for only a fraction of a second (Lauffenburger *et al*, 1987). When the flagellum rotates counterclockwise the organism swims smoothly, whilst clockwise rotation of the flagellum causes the organism to tumble, making possible a change in direction (Larsen *et al*, 1974; Simon *et al*, 1989). When smooth swimming resumes it will be in a new, randomly chosen direction (Berg & Brown, 1972). Bacterial flagellar rotation and hence bacterial motility is modulated by attractant and repellent chemotactic stimuli. Attractants increase the counterclockwise bias of flagella, with a resultant prolonged swim-

ming in rather straight lines, whilst repellents increase the clockwise bias of the flagella, resulting in tumbling (Berg & Brown, 1972; Macnab & Koshland, 1972; Larsen *et al*, 1974; Lapidus *et al*, 1988).

Chemotaxis results from a decrease in the tumbling probability for a cell moving up a concentration gradient of attractant or down a concentration gradient of repellent (Brown & Berg, 1974; Lauffenburger, *et al*, 1987). Thus when the bacterium proceeds, during a smooth swimming period, from a region of low attractant concentration to a region where attractant is more prevalent, tumbling response is suppressed and the bacterium continues to swim in the same direction. When the cell encounters decreased concentrations of attractant however, the frequency of tumbling increases. After a brief period of tumbles, the organism resumes smooth swimming until further changes in the concentrations of attractants or repellents are encountered. Thus the cell executes a biased random walk with net movement up the spatial gradient of an attractant, and vice versa in the case of a repellent (Berg & Brown, 1972; Brown & Berg, 1974). Bacteria respond to spatial gradients of attractants or repellents by monitoring the change in concentration of chemicals over time as they swim from one place to another ie swimming cells perceive spatial gradients indirectly through a chemosensor, sensing only the overall temporal change in concentration as they move in the presence of both temporal and spatial gradients (Macnab & Koshland, 1972; Lauffenburger *et al*, 1987). Monitoring concentration with respect to space would not be possible since bacteria are too small to reliably detect spatial gradients across their own dimensions (Macnab & Koshland, 1972). In nature, bacteria experience temporal concentration gradients as they swim through spatial gradients formed by diffusion from chemical sources. Immediately after the presentation of a temporal stimulus constituting an attractant, bacteria will demonstrate prolonged periods of smooth swimming, the duration of the response being proportional to the magnitude of the concentration change (Macnab & Koshland, 1972 ; Spudich & Koshland, 1975). After the initial excitation response the cell behaviour adapts,

and even in the continued presence of an attractant the tumbling frequency returns to normal (Berg & Brown, 1972; Macnab & Koshland, 1972; Spudich & Koshland, 1975). This process of desensitization or adaptation is specific for a chemoattractant since the bacteria still respond to other unrelated attractants. Adaptation is an essential part of the chemotactic response allowing bacteria to respond to changes in concentration and therefore to prolong swimming when moving in a favourable direction. In the absence of the adaptation process the bacteria would be stimulated indefinitely by just one compound and unable to respond to other, potentially nutritionally useful, attractants

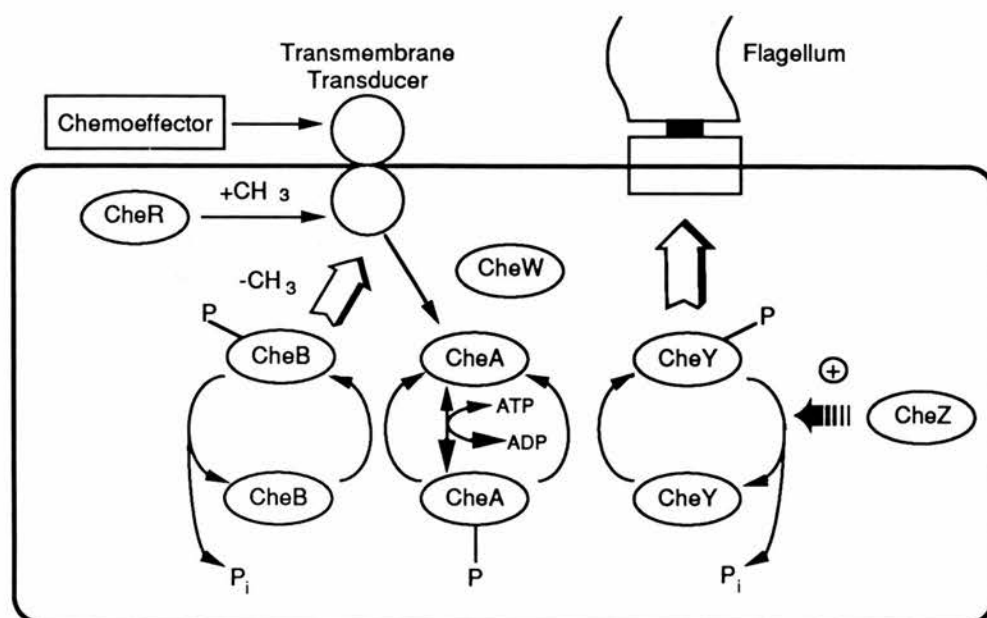
Bacteria detect chemicals in the environment by specific chemoreceptors (transducers) (Adler, 1987). About twenty different chemosensors are known for various attractants and repellents (Adler, 1987). Chemoreceptors may either be periplasmic proteins or integral parts of the cytoplasmic membrane (Hammond *et al*, 1984; Brass, 1986). Many of the latter appear to be transmembrane proteins and are proposed to have an N-terminal sensing domain located in the periplasm as well as a C-terminal signalling domain in the cytoplasm (Nixon *et al*, 1986; Bourret *et al*, 1989). For each sensor protein there is a corresponding regulator protein and it is proposed that these proteins are involved in a two component regulatory system of chemotaxis (Bourret *et al*, 1989; Simon *et al*, 1989). Each chemoreceptor is highly specific, binding only one specific chemical or its closely related analogue (Hammond *et al*, 1984). Many of the chemoreceptors serve as both receptors and help to mediate the transport of specific chemicals across the plasma membrane. Ability to move towards higher chemical concentrations is advantageous only if that compound can be efficiently transported and metabolised (Hammond *et al* 1984).

The major components of the chemotaxis information processing network of *E. coli* have been identified, although the detailed molecular mechanisms of signal transduction remain elusive (Bourret *et al*, 1989). The discovery that methionine is required for chemotaxis led to the discovery of transmembrane

proteins called methyl-accepting chemotaxis proteins (MCP) (Springer *et al*, 1979). There are four distinct MCPs known, each serving a different set of attractants and repellents: in most cases the transmembrane protein acts as a receptor for a specific ligand or ligand binding protein in the periplasmic space (Bourret *et al*, 1989). A derivative of methionine, S-adenosylmethionine, a potent methylating agent is thought to methylate MCPs. The level of methylation is increased by attractants and decreased by repellents (Springer *et al*, 1979). Protein methyltransferase, the product of *CheR* gene progressively methylates glutamyl residues in the methylation regions until the attractant-induced signal is cancelled and the cell is adapted in the presence of the attractant (Springer *et al*, 1979). Thus the level of methylation will remain at the new level as long as the attractant is present. When the attractant is removed the respective MCP is demethylated by a methylesterase, the product of the *CheB* gene, until the signal is returned to the prestimulus state (Stock & Koshland, 1978). Chemotaxis of *P. aeruginosa* towards amino acids is mediated by dynamic methylation and demethylation of MCPs analogous to those of the enteric bacteria (Craven & Montie, 1983).

Since MCPs are anchored in the cell membrane they cannot transmit the message across the inside of the cell to the flagellum. One candidate for a mediator of signalling between the receptors and the flagellar apparatus is protein phosphorylation (Hess *et al*, 1987; Bourret *et al*, 1989; Simon *et al*, 1989). ATP has been shown to be required for the chemotaxis of *P. aeruginosa* (Armitage & Evans, 1983). The excitation pathway involves a number of proteins which ultimately determine the direction of rotation of flagella. Some of these proteins, CheA and CheY can be phosphorylated and interact with the MCPs. CheA is an autophosphorylating protein kinase (Hess *et al*, 1987; Bourret *et al*, 1989). Phosphorylated CheA can then transfer the phosphoryl group to either CheB (methylesterase) or CheY (Hess *et al*, 1988). Genetic and biochemical evidence suggest that CheB and CheY proteins are activated by phosphorylation (Sanders & Koshland, 1988), thereby eliciting the excitation

a.



b.

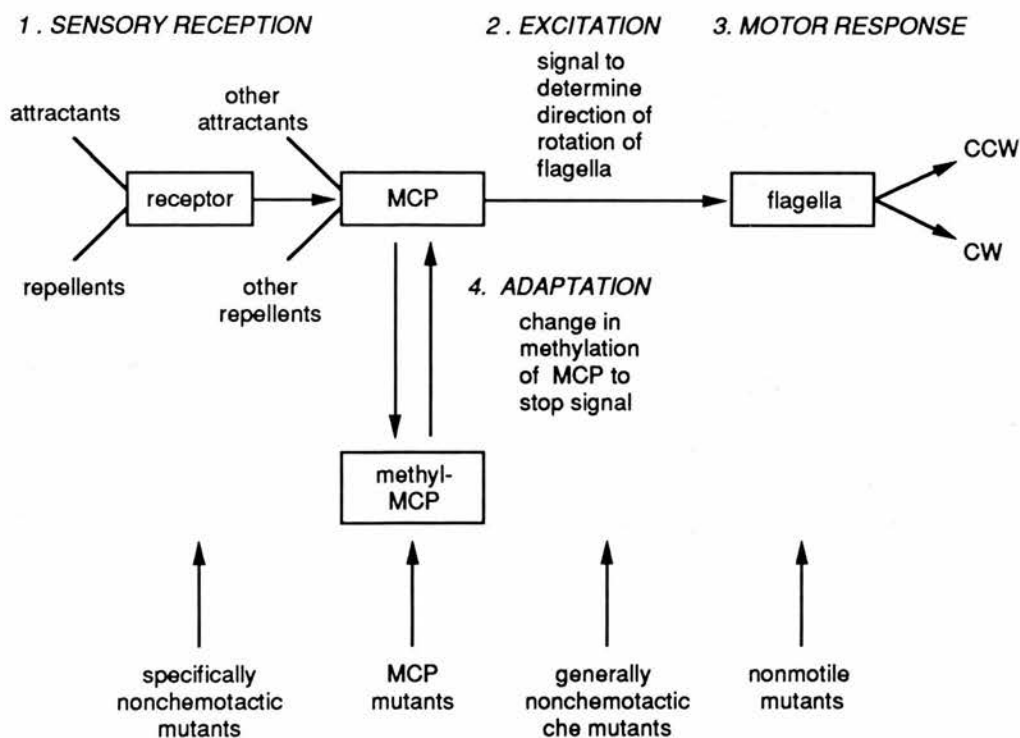


Figure 2. a) Model for the mechanism of excitation and adaptation during bacterial chemotaxis. (Figure modified from Simon *et al*, 1989, see text for description).

b) The mechanism of bacterial chemotaxis. CCW = counterclockwise rotation of the flagella; CW = clockwise rotation; MCP = methyl- accepting chemotaxis protein. (Figure modified from Adler, 1987).

response (CheY) or effecting the adaptation response (CheB) (Simon *et al*, 1989). Phosphorylation of the CheB protein would increase its methylesterase activity allowing it to change the sensibility of the receptor (Simon *et al*, 1989). It has been suggested CheY-phosphate might cause clockwise rotation of flagella and be the tumble signal (Simon *et al*, 1989). Phosphorylation is transient, and dephosphorylation of CheY is catalyzed by the CheZ protein (Hess *et al*, 1988). In addition, the receptor activated-state of another protein CheW may regulate the CheA dependent phosphorylation of CheB and CheY (Hess *et al*, 1988). A model for bacterial chemotactic signalling which incorporates the observations outlined above has been proposed (Bourret *et al*, 1989; Sanders *et al*, 1989; Simon *et al*, 1989) (Figure 2a). During the response to an increase in attractant concentration the receptors deactivate the CheW protein. CheA dependent phosphorylation of the CheB and CheY proteins is thereby reduced and results in the suppression of receptor demethylation and tumbling (clockwise flagella rotation). The attractant induced receptor conformational change increases the activity of the CheR methyltransferase and decreases the activity of the CheB methylesterase thus raising the level of receptor methylesterification and effecting adaptation. The more highly methylated receptor would activate CheW protein until its levels were high enough to once again stimulate CheA dependent phosphorylation of the CheB and CheY proteins to the level achieved before the increase in attractant concentration. An increase in CheY-phosphate induces clockwise rotation of flagella (tumbling).

Catalytic enhanced desphosphorylation of the CheY protein by the CheZ protein will counteract the excitation signal.

3.5 PILI

The pili (fimbriae) of *P. aeruginosa* have been shown to be polar located (Bradley, 1972). Pili serve as an attachment site for various *Pseudomonas* specific bacteriophages (Bradley & Pitt, 1974) and are responsible for twitching motility (Bradley, 1980). Pili may assist in the attachment of organisms to var-

ious cell surfaces: they have been reported to be involved in the binding of *P. aeruginosa* to human buccal epithelial cells (Woods *et al*, 1980b; McEachran & Irvin, 1985; Doig *et al*, 1988), acid injured mouse tracheal cells (Ramphal & Pyle, 1983a; Ramphal *et al*, 1984), tracheobronchial mucins (Ramphal *et al*, 1987) and damaged corneal epithelial cells (Reichert *et al*, 1983). Transferable plasmids have been shown to determine conjugative pili, which are also receptors for donor specific phages (Bradley, 1983).

Fimbriation is a reversible trait, most likely to be determined by chromosomal genes, and has been shown to be under the control of the *rpoN* gene product (Totten *et al*, 1990). Pili are expressed in relatively small numbers, 2-12 per pole (Bradley, 1972), and are proteinaceous appendages about 6 nm wide, composed of a single monomer termed pilin (Sastry *et al*, 1985). The pili purified from *P. aeruginosa* strain K have been extensively characterised and the primary amino acid sequence determined (Sastry *et al*, 1985). Pilin from *P. aeruginosa* K (PAK) was found to be built up of subunits of Mr 17,800, whilst the serologically distinct pili of *P. aeruginosa* O (PAO) possessed smaller subunits (Mr 15,500) (Sastry *et al*, 1985).

3.6 EXOPOLYSACCHARIDE

Despite the isolation of *P. aeruginosa* from a wide range of environmental niches and various tissue sites in infected hosts, the organism is not typically associated with a mucoid colonial morphology and alginate biosynthesis at readily detectable levels (Govan, 1988). Thus the organism tends to appear non-mucoid when cultured on an agar media (Govan, 1990). However, as previously mentioned, respiratory isolates from patients with CF frequently exhibit the mucoid phenotype (Govan, 1988). It is important at this point to make the distinction between the alginate exopolysaccharide produced by mucoid *P. aeruginosa*, and slime, produced by most *P. aeruginosa*. Slime is a loosely defined material with variable composition depending on the strain, cultural conditions, and method of analysis (Govan, 1990). The term mucoid is restrict-

ed to those strains producing the large, watery colonial type 5 of Phillips (1969) within 24 hours on agar based media, and whose mucoid appearance results from the copious production of the polyuronide, alginate (Govan, 1990).

The observation that *P. aeruginosa* was able to synthesize an alginate-like polysaccharide was first reported by Linker & Jones (1964). Alginate produced by mucoid *P. aeruginosa* is a strongly anionic polysaccharide, composed of linear 1-4 linked chains of β -D-mannuronic acid and its C5 epimer α -L-guluronic acid (Evans & Linker, 1973; Gacesa & Russell, 1990). The uronic acids are monosaccharides which have been oxidised at C6 to produce a carboxylate group and therefore are negatively charged (Gacesa & Russell, 1990). A distinctive feature of alginates from mucoid *P. aeruginosa* is that the mannuronate residues are O-acetylated (Sherbrock-Cox *et al*, 1984). The two different uronic acids may be arranged in different ways within the alginate molecule to form block structures. There may be homopolymeric regions ie poly β -D-mannuronate or poly α -L-guluronate, or heteropolymeric regions in which there is a random arrangement of the monomers (Russell & Gacesa, 1989; Gacesa & Russell, 1990). Since alginate is polyanionic it is able to bind cations, particularly of the divalent kind eg Ca^{2+} and Mg^{2+} (Russell & Gacesa, 1989).

A significant property of alginate from mucoid *P. aeruginosa* is the absence of polyguluronate blocks (Sherbrock-Cox *et al*, 1984). In addition alginates derived from *P. aeruginosa* and other pseudomonads show a predominance of the heteropolymeric random block structures. Alginate produced by mucoid *P. aeruginosa* tends to be highly viscous and flexible, with the capacity to bind large quantities of water (Gacesa & Russell, 1990). The alginate displays the ability to form rigid, stable gels in the presence of divalent cations, particularly Ca^{2+} (Rees, 1972). Culture of mucoid *P. aeruginosa* in the presence of 3 mM Ca^{2+} causes the normal mucoid colonial form to assume a more compact gelatinous appearance on agar media and the formation of bacterial microgels

which rapidly sediment in liquid culture (Govan & Harris, 1986). Electron microscopy shows the microgels as cotton-wool like meshes enclosing the bacterial cells (Govan & Harris, 1986).

Evidence obtained *in vitro* by the use of NMR spectroscopy on the polysaccharides produced by mucoid and non-mucoid isolates from both CF and non-CF patients indicates that alginate is not restricted to the mucoid colonial phenotype: non-mucoid *P. aeruginosa* and revertants of mucoid isolates have been found to produce alginate but at very much lower levels than in mucoid variants (Anastassiou *et al*, 1987). Thus the mucoid phenotype is thought to result from the derepression of a normal regulatory system resulting in a shift from low, barely detectable levels of alginate in non-mucoid strains to high levels in the mucoid phenotype (Govan, 1990).

CHAPTER 4

PATHOGENESIS OF *P. AERUGINOSA* IN CYSTIC FIBROSIS

Pathogenicity is the capacity of microorganisms to produce disease in susceptible hosts (Smith, 1977). Not all pathogens have an equal chance of causing infection and disease, and in particular instances disease occurs or is prevented depending on the outcome of interactions between the microbe and the host (Smith, 1977; Finlay & Falkow, 1989). While some pathogens regularly cause disease in a proportion of non-immune individuals with intact host defences, others, including *P. aeruginosa*, do not (Finlay & Falkow, 1989). As discussed earlier *P. aeruginosa* can infect compromised individuals and cause disease but presents little danger to those with intact host defences. Indeed probably any microorganism which has the capacity to sustain itself in humans will occasionally cause disease in compromised individuals and act as an opportunistic pathogen (Finlay & Falkow, 1989).

Essential requirements for pathogenicity are: 1) ability to enter the host; 2) multiply in host tissues; 3) resist or not stimulate host defences; 4) cause damage to the host (Smith, 1977; Finlay & Falkow, 1989; Smith, 1990). Microbial factors responsible for these processes are the determinants of pathogenicity or virulence factors, and include both cell-associated and extracellular products (Smith, 1977). Since pathogenesis is a multifactorial process, potential pathogens usually require a number of virulence factors, the absence of one member of the full complement resulting in considerable attenuation of the organism concerned (Smith, 1977).

P. aeruginosa manifests a variety of cellular and extracellular products, many of which have been implicated as virulence factors in the pathogenesis of the

organism in CF. In turn, the CF patient appears to respond to cellular and extracellular products of *P. aeruginosa* with an array of host defences (Høiby *et al*, 1975; Schiøtz, 1982) yet *P. aeruginosa* is never eliminated from the respiratory tract, nor does it spread systemically beyond this site in CF patients to cause non-pulmonary infections.

4.1 PULMONARY HOST DEFENCES

The respiratory tract possesses an elaborate array of non-specific and specific defence mechanisms which coordinate to remove a variety of noxious substances, particles and microorganisms and prevent injury to the mucosa and delicate alveolar surface (Reynolds, 1989). These protective mechanisms may be classified as follows:-

- a) Mechanical: including the cough reflex and the mucociliary clearance mechanism which is important between the posterior two-thirds of the nasal cavity and the nasopharynx, and the larynx to the terminal bronchioles (Newhouse *et al*, 1976).
- b) Anatomical: including the nose, epiglottis, larynx and respiratory branch which are responsible for directing airflow. Branching and angulation of airways are important in causing aerosolised particles to impact on the mucosa, after which mucociliary clearance and other mechanisms on the mucosal surface can aid removal or inactivation of offending particles (Reynolds, 1989).
- c) Non-specific factors: including lysozyme; anti-proteinases including α_1 -proteinase inhibitor, antileukoproteinase and α_2 -macroglobulin; iron-binding proteins, lactoferrin and transferrin; anti-bacterial activity of lung surfactant; phagocytosis by alveolar macrophages and polymorphonuclear leucocytes; bacterial cell lysis and enhancement of phagocytosis (opsonophagocytosis) by the alternative complement pathway.
- d) Specific immunological mechanisms: including the initiation of a humoral antibody and a cellular response; opsonisation of microorganisms with antibody enhances phagocytosis and initiates the classical complement

pathway involved in the antibody lysis of cells as well as the attraction and enhancement of the function of phagocytic cells.

The normal respiratory host defences outlined above combine to provide surveillance and protection of the respiratory tract under normal circumstances. However, if a sufficiently large bacterial inoculum reaches the lower respiratory tract or if particularly virulent microorganisms are inhaled, the alveolar macrophage system, for example, may be overwhelmed (Reynolds, 1983; Reynolds, 1989). In such circumstances the lung parenchyma mounts an extensive inflammatory response that may be perceived as clinical illness (Reynolds, 1983). Polymorphonuclear leucocytes may be recruited from the intravascular spaces providing a secondary supply of phagocytes. A number of factors may initiate the influx of polymorphonuclear leucocytes into the alveoli, including chemotaxins produced by alveolar macrophages, lymphokines produced by immune T-lymphocytes, and the potent leucoattractant C5a, one of the complement proteins (Reynolds, 1983; Fick, 1989). In addition, alteration of the permeability of the alveolar surface as a result of inflammation allows the entry of immunologic factors from the plasma including extra complement, additional antibodies including IgM, and other immune constituents including protease inhibitors (Reynolds, 1983; Reynolds, 1989).

4.2 INITIAL COLONISATION OF THE CYSTIC FIBROSIS RESPIRATORY TRACT

Initial colonisation by *P. aeruginosa* is an important predisposing factor in the acquisition of pulmonary infection. The transition from asymptomatic *P. aeruginosa* colonisation to chronic and progressive pulmonary infection is difficult to define and may vary from one patient to another (Høiby, 1974).

Typing of *P. aeruginosa* including pyocin typing (Fyfe *et al*, 1984; Govan, 1988), and a method based on Southern blotting and hybridisation with a DNA probe derived from the exotoxin A gene (Ogle *et al*, 1987), have proved useful

epidemiological tools and indicated the following:- 1) cross infection between patients is rare, except between CF siblings; 2) the majority of CF patients remain colonised with a single strain of *P. aeruginosa* which eventually exhibits the mucoid phenotype (Govan, 1990).

The presence of non-mucoid strains of *P. aeruginosa* in the CF respiratory tract has previously been deemed of no clinical significance (Henry *et al*, 1982). However, non-mucoid strains probably play a critical role as a microbial reservoir to allow the emergence and selection of more adaptable mucoid phenotypes associated with progressive lung disease (Govan *et al*, 1984; Govan, 1988). The time taken for the emergence of mucoid *P. aeruginosa* in individual patients is difficult to monitor since conversion is insidious and may occur in the time interval between bacteriological investigations, and in many patients both non-mucoid and mucoid forms are isolated on initial sputum culture (Govan, 1990). Epidemiological studies have indicated that the time interval between initial colonisation with a typical non-mucoid strain and the emergence of a mucoid form can be as short as three months (Govan, 1990).

4.3 SITES OF INITIAL COLONISATION

The precise mechanism of colonisation and the nature of the sites of early asymptomatic colonisation with *P. aeruginosa* remain obscure. It has been suggested that colonisation of the upper respiratory tract may precede aspiration of the organism into the lower airways where colonisation is established (Komiyama *et al*, 1985; Lindemann *et al*, 1985; Baker, 1990). Reports of upper respiratory tract colonisation sites include maxillary sinuses (Shapiro *et al*, 1982), tongue, buccal mucosa, saliva (Komiyama *et al*, 1985; Lindemann *et al*, 1985) and dental plaque (Komiyama *et al*, 1985). The difficulty of identifying oral colonisation and distinguishing it from sputum contamination because of the high concentration of bacteria in sputum was stressed by Lindemann *et al* (1985). The average age of the patients in the studies of Lindemann *et al* (1985) and Komiyama *et al* (1985) were 19 and 13.5 years respectively and therefore

likely that many of the patients had chronic *P. aeruginosa* lung colonisation. Since neither study reported examination of patients before they became sputum positive, the presence and significance of *P. aeruginosa* isolated from the oral cavity is uncertain. Indeed Iacocca *et al* (1963) reported that isolation of an organism from deep throat or nasopharyngeal swabs of ten CF patients reflects the presence of the organism below the larynx. In a recent report Taylor *et al* (1990) were unable to demonstrate the presence of *P. aeruginosa* in the upper respiratory tract of patients with an absence of pulmonary involvement, even in the case of a child who became sputum positive during the study.

Support for an oral reservoir of *P. aeruginosa* has been reported in a series of investigations by Komiyama and colleagues. Komiyama & Gibbons (1984) demonstrated interbacterial adhesion between strains of *Actinomyces viscosus* indigenous to the human oral cavity and *P. aeruginosa* of non-CF origin. In a later study interbacterial adhesion between *P. aeruginosa* (non-mucoid and mucoid) and indigenous oral bacteria (including *Streptococcus sanguis*, *Streptococcus mitis*, *A. viscosus* and *Actinomyces naeslundii*) all of which were isolated from the oral cavity of CF patients, was reported (Komiyama *et al*, 1987a). The coaggregation reactions were dependent upon heat and protease sensitive components of *Pseudomonas*, indicating that the interbacterial adhesion was mediated by adhesins on the *P. aeruginosa* cell which bind to complementary receptors on the cell surfaces of oral bacteria. It was suggested that interbacterial adhesion between *P. aeruginosa* and the oral bacteria had a possible role in oral colonisation of CF patients and thereby influenced susceptibility of the host to infection (Komiyama *et al*, 1987a).

Further studies also demonstrated the saliva-mediated aggregation of *P. aeruginosa*, the authors speculating that this mechanism may have a role in colonisation of patients with CF (Komiyama *et al*, 1987b; Komiyama *et al*, 1989). The increased aggregation of *P. aeruginosa* by saliva from patients with CF was directly related to the sialic acid content present, suggesting that this acidic

molecule acts as the salivary receptor for *P. aeruginosa* (Komiyama *et al*, 1987b). The sero-mucous products of the submandibular gland of patients with CF were shown to have enhanced *P. aeruginosa* aggregation activity, and proposed to have a role in the mechanisms by which the organism colonises or is cleared from the oral cavities of patients with CF (Komiyama *et al*, 1989).

The possibility that colonisation of the gastrointestinal tract might act as a reservoir of non-mucoid *P. aeruginosa* in CF has also been investigated (Agnarsson *et al*, 1989). Faecal isolation of *P. aeruginosa* was observed in eight of ten patients who at the time of sampling exhibited colonisation of the respiratory tract. Thus in this group of patients it was not possible to determine whether gut colonisation preceded, or resulted from, colonisation of the respiratory tract. In contrast, *P. aeruginosa* cells were isolated at low frequency (9.1%) from the stools of 44 patients with no previous history of chronic colonisation. The incidence for this latter group of CF patients was within the normal range for both non-hospitalised patients (Rhame, 1980) and the 20-54% isolation rate reported for hospitalised patients (Bodey, 1970, Young & Armstrong, 1972). These observations led the authors to conclude that the gastrointestinal tract is not a significant reservoir of *P. aeruginosa* prior to pulmonary colonisation.

4.4 THE ROLE OF ADHERENCE IN COLONISATION

The first major interaction between a potential pathogenic microorganism and its host, entails attachment to a eukaryotic cell surface (Finlay & Falkow, 1989). Some microorganisms multiply at and remain on the surface whilst others use attachment as the first essential step before proceeding to deeper tissue or other locations. Adherence of *P. aeruginosa* to a number of cell surfaces has been reported and implicated as one of the factors for colonisation and establishment of the organism in the CF respiratory tract.

Adherence to buccal epithelial cells

An initial *in vitro* study on the adherence of *P. aeruginosa* revealed adhesion of the organism to buccal epithelial cells (Johanson *et al*, 1979). Enhanced adhesion was observed with buccal cells obtained from non-CF patients colonised with Gram-negative bacilli or after prior treatment of buccal cells with trypsin (Johanson *et al*, 1979). Inhibition of adherence by purified pili and anti-serum to pili indicated the role of pili in adherence of *P. aeruginosa* to buccal epithelial cells (Woods *et al*, 1980b). Subsequent *in vitro* studies showed adherence of *P. aeruginosa* to buccal cells obtained from patients with CF, increased adherence being associated with decreased amounts of the cell surface glycoprotein, fibronectin, and increased levels of salivary proteases (Woods *et al*, 1980a; Woods *et al*, 1981). Normal resistance of upper respiratory tract epithelial cells to Gram-negative colonisation is believed to be associated with the presence of fibronectin (Woods *et al*, 1983). Fibronectin is thought to mask Gram-negative bacterial receptor sites on the epithelial cell surface. Since fibronectin is highly sensitive to proteases such as trypsin (Woods *et al*, 1981) and since salivary protease levels in CF patients are significantly elevated over those of controls, it would be reasonable to assume that increased levels in secretions which bathe mucosal surfaces, may be responsible for the decreased levels of fibronectin exposing receptors for adhesion to buccal cells (Woods *et al*, 1980a).

Although these *in vitro* observations suggested that buccal cells may form the reservoir for colonisation of the CF respiratory tract this step has been difficult to observe *in vivo* and would require frequent screening of patients. *In vivo* observations reported by Govan (1988) showed that *Pseudomonas* was cultured on only two occasions from buccal cells obtained directly from 88 CF patients. Indeed a comparison of the adherence of *P. aeruginosa* to ciliated cells and buccal cells revealed that nasal and tracheal cells bound more bacteria per cell than did buccal cells (Niederman *et al*, 1983). More recent *in vitro* studies have further demonstrated pilus mediated adhesion of non-mucoid *P. aeruginosa* to buccal epithelial cells (Doig *et al*, 1988; Doig *et al*, 1990). Investigations into the

nature of the receptors indicate that they may consist of carbohydrate moieties. Adherence of *P. aeruginosa* and *P. cepacia* to glycolipids on thin-layer chromatograms demonstrated binding to gangliotetraosylceramide (asialo GM1) and gangliotriasylceramide (asialo GM2) (Krivan *et al*, 1988). In addition Baker (1990), and Baker *et al* (1990) reported adherence of *P. aeruginosa* to a number of sialylated glycolipids. Terminal or internal N-acetyl- β -D-galactosamine (1-4) β -D-galactose sequences were implicated as the minimal, specific binding site (Krivan *et al*, 1988). Studies by Doig *et al* (1989) showed that periodate oxidation of buccal epithelial cell blots eliminated pilus binding, indicating that a carbohydrate molecule was necessary for receptor activity. Sugar inhibition studies showed that sialic acid and L-fucose prevented pilus binding to the buccal cells.

The initial adhesion studies by Woods *et al* (1980a) indicated that mucoid strains did not adhere to normal or trypsinated buccal cells. This difference in adhesion between non-mucoid and mucoid strains was thought to possibly account for the common observation that initial *Pseudomonas* colonisation of the CF respiratory tract was by non- mucoid *P. aeruginosa* (Woods *et al*, 1980a). However, more recent evidence has shown that mucoid strains could also adhere to buccal cells (McEachran & Irvin, 1985). Two classes of receptors were identified:- 1) a high affinity, low copy number site, present after trypsination and hypothesized to be the receptor for pili; 2) a low affinity, high copy number, trypsin sensitive site proposed to be the receptor for alginate (McEachran & Irvin, 1985). In a later study the binding of mucoid *P. aeruginosa* to buccal and tracheal epithelial cells was shown to be strain dependent reflecting structural diversity in *P. aeruginosa* alginates (Doig *et al*, 1987).

Adherence to cells of the lower respiratory tract

Initial colonisation of *P. aeruginosa* in the upper respiratory tract has been proposed to precede aspiration into the lower respiratory tract where they colonise epithelial cells or tracheobronchial mucins (Baker, 1990). Adherence

studies of *P. aeruginosa* to hamster tracheal explants revealed that mucoid organisms were more adherent than the non-mucoid isolates (Baker & Marcus, 1982). Mucoid organisms were usually found in microcolonies adhering to cilia, transmission electron microscopy revealing interaction of the bacterial glycocalyx with the glycocalyx on the cilia (Baker & Marcus, 1982). Quantitation of adherence by the tracheal perfusion model further indicated the greater affinity of mucoid strains of *P. aeruginosa* for tracheal epithelium isolated from hamsters, mice and guinea pigs (Marcus & Baker, 1985; Marcus *et al*, 1989). Scanning electron microscopy again revealed the presence of microcolonies of mucoid organisms attached to cilia and associated with the mucus layer covering the epithelium (Marcus & Baker, 1985). Adherence of mucoid isolates to cilia of human tracheal cells has also been observed (Franklin *et al*, 1987).

A number of experimental observations indicated that the adhesin for mucoid strains was alginate including inhibition by the positively charged polymer poly-L-lysine, inhibition by anti-alginate antibodies (Marcus *et al*, 1989; Baker, 1990), and by the ability of purified alginate to bind to the cilia of human epithelial cells (Doig *et al*, 1987). Sugar inhibition studies with the perfused-trachea model showed that N-acetylglucosamine, galactose and N-acetylneuraminic acid were the best inhibitors of mucoid strains of *P. aeruginosa* and may represent important components of receptors on cilia or mucins (Marcus *et al*, 1989).

Adherence to damaged epithelium

Ramphal & Pyle (1983a) have shown that both non-mucoid and mucoid strains of *P. aeruginosa* could adhere to acid-injured tracheal epithelium from mice. Previous work also showed that the organisms could adhere to tracheal epithelium injured by influenza virus or by intubation (Ramphal *et al*, 1980). Additional studies using the acid-damaged tracheal model showed that the adherence of mucoid and non-mucoid strains was inhibited by N-acetylneuraminic acid and mucin (Ramphal & Pyle, 1983b). Purified pili and anti-pilus antibodies inhibited the adherence of non-mucoid strains to injured epithelium,

but had no effect on adherence of mucoid strains (Ramphal *et al*, 1984). In contrast antibodies against alginate inhibited the binding of mucoid strains to damaged epithelium but had no effect on the adherence of non-mucoid strains (Ramphal & Pier, 1985). Further studies on the tracheal receptor for *P. aeruginosa* indicated that both non-mucoid and mucoid strains bound to sialic acid containing glycolipids, probably a ganglioside (Ramphal & Pyle, 1985; Krivan *et al*, 1988).

Whilst the specific interaction between injured tracheal cells and *P. aeruginosa* is apparent, it is uncertain whether this is necessarily relevant to chronic colonisation in CF (Ramphal & Vishwanath, 1987). It is possible to speculate that cells injured by viruses or mycoplasmas during exacerbations could become colonised. In support of this theory Kubesch *et al* (1988) reported greater binding of *P. aeruginosa* to buccal cells from patients who had acute upper airway viral infection of their upper airways. Non-mucoid strains of *P. aeruginosa* do not adhere well to normal ciliated epithelial cells (Ramphal & Pyle, 1983a) indicating that prior damage may be necessary for colonisation. Morphological studies of the respiratory epithelium of CF patients who had not had any documented infections indicated that the epithelium was not abnormal (Sturgees, 1981).

Mucociliary clearance and adherence to mucins

The mucociliary transport apparatus of the respiratory tract is responsible for clearing natural cellular debris and inhaled particulate material, including bacteria, from the respiratory mucosa and is thought to play a major role in prevention of respiratory infections (Reynolds, 1983). The two major components of this mechanism are the mucus blanket and the underlying cilia whose beating motion provides the means for mucus flow up the respiratory tract.

Mucus is a complex secretion from secretory cells of the submucosal layer and surface epithelium, locally produced substances and tissue fluid transudate

(Lopez-Vidriero, 1981; Reynolds, 1983). Mucus is composed of 95% water with the glycoprotein, mucin as the major macromolecular component (Lopez-Vidriero, 1981). Other components of respiratory mucus include lipids, immunoglobulins, lactotransferrin, nucleic acids, proteolytic enzymes and protease inhibitors (Roussel, 1984). Respiratory mucins, secreted by the submucosal glands and goblet cells of the surface epithelium (Rose *et al*, 1984), are responsible for the rheological and sticky properties of mucus (Lopez-Vidriero, 1981). These high molecular mass, filamentous glycoproteins contain several hundred oligosaccharide chains in glycosidic linkages to serine and threonine residues of the polypeptide chain (Rose *et al*, 1984; Slayter *et al*, 1984). The highly glycosylated regions are resistant to proteases, whilst the naked regions devoid of carbohydrate chains are susceptible to proteolytic degradation.

The question of an inherited abnormality in mucins from patients with CF is uncertain although increased levels of sulphation of the carbohydrate chains has been reported (Boat *et al*, 1976; Chace *et al*, 1985). Some characteristic features of CF mucin including increased acidity and a decreased proportion of neutral glycopeptides (Houdret *et al*, 1989) and the apparent lower molecular mass of CF respiratory mucins (Rose *et al*, 1987) have been attributed to ^{de}gradation by *P. aeruginosa*, including degradation by both *P. aeruginosa* and neutrophil proteases (Rose *et al*, 1987; Houdret *et al*, 1989; Poncz *et al*, 1988). In addition both elastase and alkaline proteinase from *P. aeruginosa* have been shown to stimulate mucin secretion by rabbit tracheal epithelium (Klinger *et al*, 1984). Since the increased production of mucin and production of viscid respiratory secretions are basic characteristics of CF and play an important part in its pathophysiology, the influence of bacterial and other proteases may exacerbate the condition.

In healthy individuals mucociliary clearance is mediated by a complex series of interactions between mucus, cilia and the intervening watery periciliary fluid lying below a thick layer of mucus (Greenstone & Cole, 1985). Mucus is re-

leased onto the surface of the ciliated epithelium as droplets and these are drawn out into strands by the action of the cilia which beat in the periciliary zone of fluid of low viscosity (Sleigh, 1981). Driven by the coordinated beating of the cilia the mucous blanket is moved at a rate of 0.5-1.5 cm/min towards the larynx where the mucus is either swallowed or expectorated (Greenstone & Cole, 1985, Hingley *et al*, 1989). If the periciliary zone of fluid is too shallow or too deep the propulsion of mucus will be severely restricted or may cease altogether (Sleigh, 1981). In addition, if the mucus layer is too thick uncoupling may occur within it so that the inner part is transported by ciliary beating but the outer part, to which bacteria may adhere, remains stationary (Cole & Wilson, 1989). In general, both the rate of ciliary beat and proportion of ciliated cells in the epithelium increase from smaller to larger airways so that the role and efficiency of mucus propulsion increases as it ascends the mucociliary escalator (Sleigh, 1981).

Malfunction of mucociliary clearance may result from intrinsically defective cilia, from infections that are ciliolytic (*Mycoplasma pneumoniae*) or actually destroy the cilia-containing epithelial cells (viral agents), and from abnormal mucus secretion (Reynolds, 1983). The term ciliary dyskinesia is used to describe congenital abnormalities in the structure and function of cilia. Patients suffering from such a condition are prone to recurrent chest infections, and coughing is a prominent symptom in an attempt to remedy mucus stasis caused by impaired mucociliary clearance (Greenstone & Cole, 1985).

In CF, mucus secretions are copious, inspissated and stagnant, mucociliary clearance is slowed and mucopurulent plugging of peripheral airways forms part of the early pathologic lesion (McPherson & Dormer, 1987; Fick, 1989). The stagnation of mucus in CF implies a defect or interference in the mucociliary system. The presence of an increased depth of tenacious and dehydrated mucus, as well as dehydrated periciliary fluids may contribute to reduced mucociliary transport in CF (Wood *et al*, 1976; Fick, 1989). Wood *et al* (1976)

reported that mucociliary clearance was five to ten-fold lower than in normal controls. A reduced mucus transport rate in 14 adult CF patients was also reported by Wanner (1981) although no correlation between transport rate and clinical status was found. However, Yeates *et al* (1976) observed abnormal mucociliary transport in some, but not all CF patients, and Rutland & Cole (1981) noted that *in vitro* ciliary beat frequency of nasal epithelium was normal in patients with CF. The presence of a ciliotoxic factor in the serum of patients with CF has been described, but is also present in unaffected heterozygotes (Spock *et al*, 1967).

Viral and mycoplasmal infections may damage the mucociliary system in CF, and inactivation of ciliary beat by extracellular products of *P. aeruginosa* and *H. influenzae* has also been reported (Wilson *et al*, 1985). In particular *P. aeruginosa* rhamnolipid, pyocyanin and proteases have been implicated as important ciliostatic factors released by *P. aeruginosa* which act on the membrane and the axoneme of respiratory cilia (Hingley *et al*, 1989; Wilson *et al*, 1989). By interfering with normal ciliary function these ciliostatic factors might enable *P. aeruginosa* to more easily colonise the respiratory tract (Hingley *et al*, 1989).

Microorganisms may become entrapped in mucus (prior to subsequent removal by cephalad ciliary movement in normal individuals) because of its stickiness or because of a specific interaction between receptors in mucin and surface components of bacteria (Vishwanath & Ramphal, 1985).

P. aeruginosa has been shown to adhere to mucin strands in mouse trachea (Ramphal & Pyle, 1983a). A microtitre plate assay developed by Vishwanath & Ramphal (1984) for the study of adherence of *P. aeruginosa* to human tracheo-bronchial mucin demonstrated that *P. aeruginosa* binds preferentially when compared to other Gram-negative organisms. Adherence of both mucoid and non-mucoid strains was reported (Vishwanath & Ramphal, 1984) and, as for

other adherence studies, the adhesins appeared to be different. Purified alginate attached to mucins and antibodies to the exopolysaccharides, blocked adherence of mucoid strains to mucin (Ramphal *et al*, 1987). Purified pili and antibodies to pili blocked adherence of non-mucoid, but not mucoid strains to mucins (Ramphal *et al*, 1987). The adherence of both groups of organisms was sensitive to periodate oxidation, to neuraminidase treatment of mucin, and to pretreatment of the bacteria with N-acetylneuraminic acid or N-acetylglucosamine (Vishwanath & Ramphal, 1985). These amino-sugars were proposed to form important constituents of the binding sites for *P. aeruginosa* on human tracheobronchial mucin (Vishwanath & Ramphal, 1985). A recent report by Ramphal *et al* (1989) suggested that CF mucins may be altered after infection with *P. aeruginosa* is established, resulting in less binding to some fragments. Sulphated fractions showed no binding of *P. aeruginosa* leading to the suggestion that sulphation may be a mechanism to reduce binding (Ramphal *et al*, 1989).

Since the clearance of mucus is defective in CF, binding of *P. aeruginosa* to mucins may play an important step in colonisation of the CF respiratory tract (Ramphal & Vishwanath, 1987). The association of *P. aeruginosa* with mucin and its relevance to colonisation in CF has been supported by other observations: Simel *et al* (1984) and Jeffery & Brain (1988) observed the association of *P. aeruginosa* with surface mucous secretions rather than with the underlying epithelia in scanning electron microscopy studies of CF lung tissue. Indeed the binding of *P. aeruginosa* to mucin has been hypothesized to protect the organism from opsonophagocytic killing and may be responsible, in part, for its persistence in the CF respiratory tract (Ramphal & Vishwanath, 1987).

4.5 ADAPTATION OF *P. AERUGINOSA* TO THE LUNG ENVIRONMENT

The ability of *P. aeruginosa* to colonise and cause significant morbidity in CF patients is due to a combination of innate and adaptive properties (Govan,

1990). As indicated previously, the isolation of mucoid *P. aeruginosa* in sputum is almost diagnostic of CF in adolescent patients with chronic pulmonary disease, and is the major cause of morbidity and mortality (Høiby, 1984; Govan, 1988; Govan & Glass, 1990).

The possibility that environmental factors within the CF bronchial secretions might induce or switch on the production of alginate and hence influence the emergence of mucoid *P. aeruginosa* has been proposed and several attendant factors proposed: the presence of dipalmitoyl-phosphatidylcholine, the major lung surfactant (Govan, 1975); increased osmolarity (Berry *et al*, 1988; Govan, 1990); effects of individual cations (Boyce & Miller, 1982a); nutrient limitation (Govan, 1990; Speert *et al*, 1990); selection by phage (Govan, 1975) and antimicrobial pressure (Govan, 1976; Govan & Fyfe, 1978; Deretic *et al*, 1986).

P. aeruginosa is associated with intrinsic resistance to many antimicrobial agents, a property held responsible for the emergence of the species as a significant opportunistic pathogen (Govan, 1989). However, in the CF lung, isolates of *P. aeruginosa* may exhibit the paradoxical characteristic of antibiotic hypersusceptibility (May & Ingold, 1972; Irvin *et al*, 1981; Fyfe & Govan, 1984). The degree of enhanced susceptibility is most evident with carbenicillin and becomes less significant as the anti-pseudomonal activity of the antibiotic increases: with ciprofloxacin, hypersusceptible variants are only 2.5 fold more susceptible than normal isolates (Govan, 1989).

Although alginate biosynthesis and hypersusceptibility are frequently present in the same isolate, the two chromosomal mutations (*bls* and *tps*) which encode hypersusceptibility are distinct from those involved in alginate biosynthesis (Fyfe & Govan, 1984). Hypersusceptibility is almost exclusively confined to respiratory isolates of *P. aeruginosa* (Irvin *et al*, 1981), the frequency of such isolates increasing in patients with established pseudomonal colonisation (Govan, 1989). Paradoxically, hypersusceptible *P. aeruginosa* are not

eliminated during antibiotic therapy and may even increase during anti-pseudomonal therapy (Govan, 1989). Hypersusceptibility together with alginate biosynthesis must confer significant advantages for bacterial growth and survival in the respiratory environment (see 4.6 The Microcolony Mode of Growth).

Another common feature of *P. aeruginosa* strains isolated from patients with CF is that of serum sensitivity (Thomassen & Demko, 1981; Hancock *et al*, 1983; Penketh *et al*, 1983). In a survey of 109 CF isolates, 87 sputum isolates from non-CF patients and 118 isolates from other clinical sources, Penketh *et al* (1983) showed that 53.2% of the CF isolates were serum sensitive which was significantly greater than in either of the control groups. In addition 68% of the CF strains could not be serotyped due to loss of the O-type reaction and 50.5% showed multiple agglutination reactions with O-antisera (ie polyagglutinable) and expressed the polyagglutinating (PA) antigen. Although there was close correlation between serum sensitivity, loss of O-type reaction and expression of PA antigen (Penketh *et al*, 1983; Pitt *et al*, 1986), some intermediate strains were identified with some, but not all of these features (Penketh *et al*, 1983). In addition these phenotypic characteristics were also seen in non-CF isolates suggesting that they are not specific to CF, but perhaps relate to chronic colonisation by the organism.

The above study also revealed that patients recently colonised by *P. aeruginosa* had strains of the organism similar in characteristics to those from other clinical sources. In contrast, patients chronically colonised by the organism tended to express all three characteristics, whilst patients infected by organisms of an intermediate type were often more severely affected by the disease. These observations led the authors to suggest that in chronic bronchopulmonary infection with *P. aeruginosa* in CF the organism undergoes an environmental adaptation marked by changes in somatic antigens and serum sensitivity, and that during the period of adaptation the condition of some patients may greatly

deteriorate.

In a similar study by Hancock *et al* (1983) a strong correlation between the apparent numbers of O-side chains, sensitivity to serum, and non-typability was reported for isolates from patients with CF. The relationship between polyagglutinability and loss of serum resistance in *P. aeruginosa* strains from CF was further reported by Pitt *et al* (1986). Evidence for the role of LPS in serum resistance was the finding that the LPS of serum resistant variants derived from serum-sensitive parents were heterogenous and contained O-side chains, whilst serum-sensitive counterparts contained primarily lipid A/core polysaccharide components (Schiller *et al*, 1984). Fomsgaard *et al* (1988a) showed immunochemical similarities between LPS core/lipid A antigens in typable and polyagglutinable strains from CF lungs indicating that this region of the molecule is conserved when O-antigenic units are lost. However, LPS integrity may not be the only factor in determining serum resistance in *P. aeruginosa*. Pitt (1989) reported that a number of IATS serotype strains as well as some serotypable clinical isolates were serum sensitive and suggested that a 32-kilodalton outer membrane protein previously identified by Schiller *et al* (1984) may play a role in serum killing.

The role of antibody and both the classical and alternative complement pathways in serum killing of serum sensitive strains of *P. aeruginosa* have been investigated. Whether complement-mediated killing is a normal host defence in sputum is unknown since many of the complement proteins are difficult to identify in respiratory secretions (Fick, 1989). Several investigators have presented evidence suggesting the importance of the classical complement pathway activated by antibodies in the killing of *P. aeruginosa* strains (Thomassen & Demko, 1981; Borowski & Schiller, 1983). Other reports have stated that certain strains are killed by complement activated via the alternative complement pathway in the apparent absence of antibodies (Pier & Ames, 1984): lipid A/core polysaccharide is known to activate the alternative complement path-

way in the absence of antibody (Morrison & Kline, 1977). The exposure of these components in O-side chain deficient strains of *P. aeruginosa* perhaps counts for their serum sensitivity. In an examination of seven serum sensitive strains obtained from the sputa of patients with CF, Schiller (1988) found that bactericidal killing could occur via either of the complement pathways, although maximum killing of four of these strains required the function of both pathways. In addition, antibodies in pooled normal human serum specific for rough-type LPS on serum-sensitive strains are able to mediate bacterial killing via the classical complement pathway (Schiller, 1988).

Antigenic variation of *P. aeruginosa* in the respiratory tract may well suppress the development of an effective antibody response to the organism (Penketh *et al*, 1983) whilst the high incidence of serum sensitive strains could account for the low incidence of septicaemia in CF patients (Hancock *et al*, 1983). However, Thomassen & Demko (1981) demonstrated that some CF sera contained blocking antibodies which neutralised the bactericidal activity of a patient's serum for the homologous *P. aeruginosa* strain.

Engels *et al* (1985) showed that with respect to *P. aeruginosa* strains PAC1 and PAC605, the lack of an O-side chain in the latter resulted in increased sensitivity to serum and decreased virulence. The authors concluded that the structure of the O-antigen polysaccharide side-chain of LPS is of crucial importance for evasion of host defence mechanisms by *P. aeruginosa*, able to resist serum killing by shedding the C5b-9 complement complexes even in the presence of antibodies. Indeed, studies by Cryz *et al* (1984) showed that an LPS-defective, serum sensitive variant was approximately 1,000 fold less virulent than the parent strain. However, the ability of an opportunistic organism like *P. aeruginosa* to modify the LPS structure, perhaps under the influence of environmental factors (Day & Marceau-Day, 1982), may give the organism a survival advantage and help it persist within the CF lung.

4.6 THE MICROCOLONY MODE OF GROWTH

Direct microscopic examination of tissue from acute *P. aeruginosa* infections, including burn and acute lung infections, reveals the organism to be widely dispersed throughout the specimen (Costerton *et al*, 1990). In contrast the mucoid *P. aeruginosa* present in the CF-post mortem lung (Lam *et al*, 1980) and in CF sputum (Høiby, 1982; Govan & Harris, 1986) can often be seen as large microcolonies adhering to bronchial mucosa. In the study of Lam *et al* (1980) direct electron microscopic examination of post mortem lung material from CF lungs revealed large, coherent microcolony structures in which alginate occupied more space than the enclosed *P. aeruginosa* cells themselves. Further experiments showed that the microcolonies could be duplicated in *in vitro* cultures and in animal model experiments involving pulmonary infections of rats by the agar bead method developed by Cash *et al* (1979).

The microcolony form of growth, also applicable for growth of *P. aeruginosa* in natural and other pathogenic environments (Costerton, *et al*, 1990), has been used to construct a model which takes account of the unusual properties of *P. aeruginosa* and the survival and pathophysiology of pseudomonal infection in the CF lung (Govan & Harris, 1986; Govan, 1988) (see Figure 3). Once initial adhesion to a surface is complete the adherent cells produce large amounts of alginate and divide to produce polysaccharide enclosed microcolonies of sister cells. A coherent biofilm is eventually produced when cell division and alginate production cause adherent microcolonies to coalesce, the process being further accelerated by adhesion of motile, planktonic bacterial cells. Organisms within the glycocalyx matrix are protected from external antibacterial influences although motile, planktonic cells may leave the biofilm and adhere to new surfaces and initiate the formation of new biofilm communities (Costerton *et al*, 1990).

The microcolonies observed in CF patients may result from the gelling potential



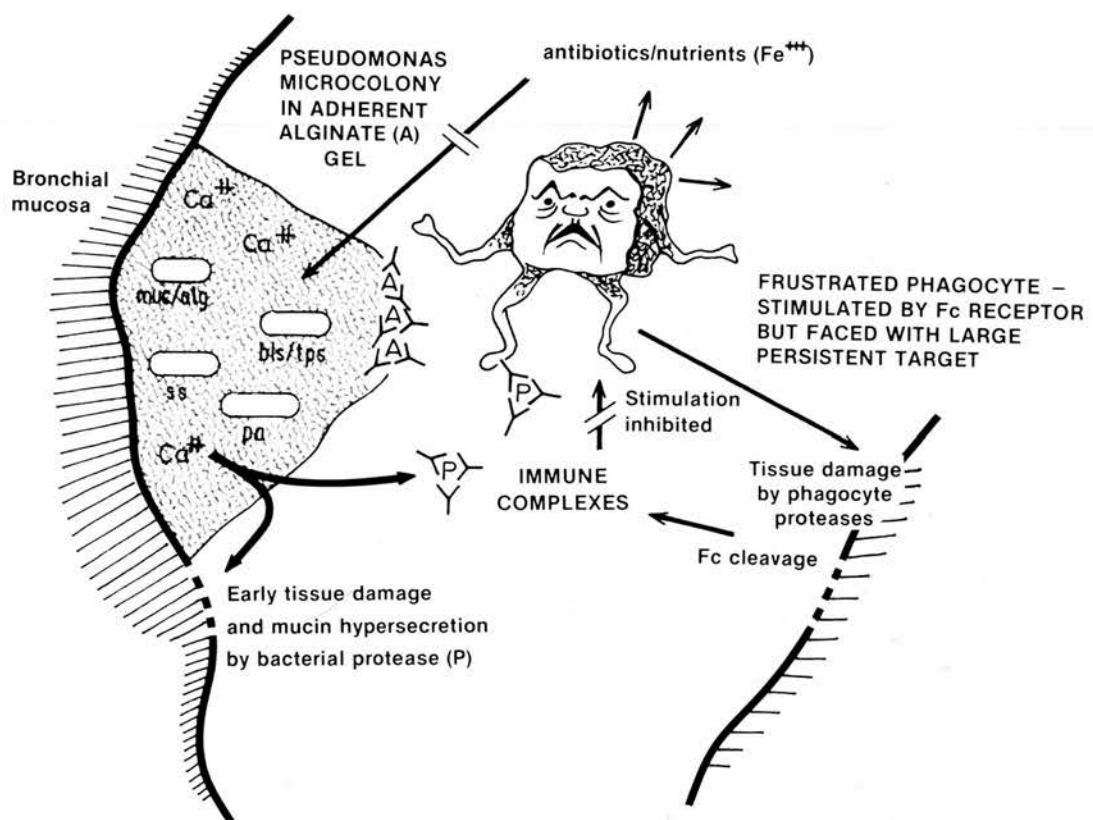


Figure 3. Microcolony mode for survival and pathogenesis of mucoid *P. aeruginosa* within a CF lung. (See text for description; from Govan, 1988). ss = serum sensitivity; pa = polyagglutinating antigen; A = alginate matrix; P = protease; muc = mucoid phenotype; alg = alginate.

of the pseudomonal alginate coupled with the raised Ca^{2+} and DNA levels found in the CF lung (Govan & Harris, 1986; Govan, 1988). As discussed earlier, the alginate may be responsible for adherence of mucoid *P. aeruginosa* to tracheal epithelium or mucosal material. Indeed, provided the bacterial alginate is not removed by centrifugation and washing, evidence from intratracheal inoculation into rat lungs indicates that mucoid *P. aeruginosa* are removed less rapidly than isogenic non-mucoid strains (Govan *et al*, 1983).

It has been proposed that motility would not be a major factor in the survival of pathogens within the microcolony (Costerton *et al*, 1990). In the case of the microcolonies found in CF this theory is supported by the observations of Luzar *et al* (1985) who reported that rough strains from patients in poor clinical condition either lacked flagella, as determined by electron microscopy, or demonstrated weak motility both in soft agar and by the capillary assay. Alterations in the synthesis of LPS were proposed to favour non-flagellated *P. aeruginosa* strains in patients who had harboured the pathogen for prolonged periods of time, since a basal body ring of the flagellum is associated with the LPS in the outer membrane of the cells.

Within the microcolony the decreased susceptibility of mucoid *P. aeruginosa* to antibiotics (in the absence of *bls* and *tps* mutations) is probably not due to a diffusion barrier or binding by the alginate but possibly from antagonism of β -lactams and aminoglycoside antibiotics by the high electrolyte content of the alginate gel structure (Slack & Nichols, 1981), modification of the alginate gel structure, or other biofilm associated mechanisms (reviewed by Govan, 1989).

Protection afforded by the microcolony may also account for the apparently paradoxical characteristics of serum sensitivity and hypersusceptibility to antibiotics. *In vivo* the selective advantage for the *bls* and *tps* mutations may lie in conferring greater permeability for the limited nutrients within the alginate matrix (Govan & Harris, 1986; Govan, 1988).

The microcolony mode may account for the inability of the normally highly efficient pulmonary immune system to remove *P. aeruginosa* from the CF lung (Govan, 1988). With other exopolysaccharide producing pathogens, including *Streptococcus pneumoniae* and *Klebsiella pneumoniae* opsonisation by specific antibodies assists ingestion and killing by phagocytic cells. However, in the CF lung the size and gelatinous nature of the alginate microcolony would present a formidable and persistent target (Govan, 1988). It has been estimated that microcolonies may reach 60 μm in diameter and are thus considerably larger than the surrounding phagocytic cells (10 μm) Govan, 1990).

The possible antiphagocytic nature of alginate has been reported by a number of investigators although the subject is controversial and has been disputed by others. In a study by Schwartzmann & Boring (1971) alginate containing extracellular material from mucoid *P. aeruginosa* inhibited the killing of bacteria by rabbit neutrophils, whilst Oliver & Weir (1983) reported that alginate inhibited the binding of an isogenic non-mucoid revertant to mouse peritoneal and pulmonary macrophages. Experiments by Simpson *et al* (1988) showed that purified exogenous alginate inhibited both the uptake and subsequent degradation of non-mucoid *P. aeruginosa* by mouse peritoneal macrophages. The authors concluded that evasion of host defences is probably due to the physical rather than the chemical properties of the alginate, including both size and viscosity characteristics. Further evidence presented by Simpson *et al* (1989) demonstrated that the scavenging properties of *P. aeruginosa* alginate may protect the bacterium from free radicals generated by phagocytes and hence interfere with the killing mechanisms of phagocytes.

Both Cabral *et al* (1987) and Krieg *et al* (1988) showed that alginate from mucoid *P. aeruginosa* may render the organism resistant to non-opsonic phagocytosis, the former suggesting that this property may give a survival advantage in the early course of pulmonary infection before opsonic antibody

and complement are present in respiratory secretions.

Baltimore & Mitchell (1980) found that a higher concentration of antiserum was required to achieve a ten-fold reduction in viable mucoid *P. aeruginosa* compared to isogenic non-mucoid strains. However, these observations were not confirmed by LeBlanc *et al* (1982) who could find no difference between mucoid and non-mucoid strains in a chemiluminescence assay with CF serum. Indeed, the antiphagocytic effect ascribed to mucoid exopolysaccharide was not confirmed by Pier (1989) who suggested that variation in technique and source of assay components was likely to account for some of the discrepancies.

The term "frustrated macrophage" has been applied to phagocytes which would normally phagocytose *P. aeruginosa* (Govan & Harris, 1986; Govan, 1990). Frustrated phagocytic cells, particularly pulmonary neutrophils stimulated by immune complexes or altered immunoglobulins, release elastase and toxic oxygen radicals which may explain the considerable evidence for immune mediated damage in CF lungs (see Chapter 5).

4.7 EXTRACELLULAR VIRULENCE FACTORS OF *P. AERUGINOSA* AND THEIR ROLES IN PATHOGENESIS OF CYSTIC FIBROSIS

Indirect evidence for the production *in vivo* of exoenzymes by CF strains of *P. aeruginosa* have come from the observation that significant levels of antibodies to the bacterial exoenzymes have been detected in sera of infected patients (Granstrom *et al*, 1984; Doring *et al*, 1985; Hollsing *et al*, 1987). In addition, many CF isolates of *P. aeruginosa* are able to produce the respective exoenzymes *in vitro* (Woods & Sokol, 1986).

P. aeruginosa produces two proteases alkaline phosphatase and elastase, both of which are metalloproteases (Kharazmi, 1989). There is considerable evidence from *in vitro* studies that *P. aeruginosa* proteases inhibit the function of phagocytes, natural killer cells and T cells, probably by cleavage of receptors involved

in cell function. The proteases also inhibit the biological activities of interleukin-1 and interleukin-2, and inactivate immunoglobulins and complement (reviewed by Kharazmi, 1989). Baker (1982), utilising hamster tracheal organ culture, concluded that the elastase (and other exoenzymes) of *P. aeruginosa* may be responsible for much of the destruction of respiratory tissue in *Pseudomonas* infections. In CF, *P. aeruginosa* elastase has been implicated in the cleavage of IgG, secretory IgA and the complement proteins C3 and C5, and therefore hinders opsonophagocytosis of the organism (Fick, 1989; Bainbridge & Fick, 1989).

The bulk of the proteolytic activity in bronchial secretions has been attributed to the neutrophil serine elastases including elastase, collagenase and cathepsin G (Suter *et al*, 1984). However, although the *Pseudomonas* elastase contribution to the total proteolytic activity present in CF airway fluids is small (Suter *et al*, 1984), the potency of *P. aeruginosa* elastase for the substrate elastin far exceeds that of human neutrophil elastase, and the bacterial exoprotein is likely to be present in higher concentrations in the local milieu immediately surrounding the microcolony (Bainbridge & Fick, 1989). In addition, the major proteinase inhibitors of the human airway, α_1 -proteinase inhibitor and antileukoproteinase are inactivated by the bacterial proteinase (Fick, 1989). The only airway proteinase inhibitor with reported neutralizing activity versus *Pseudomonas* elastase is α_2 -macroglobulin which is usually present at only very low levels in airway secretions (Bainbridge & Fick, 1989).

The exoproteinases of *P. aeruginosa* are believed to support bacterial colonisation at the onset of infection by impairing antimicrobial defence mechanisms of the CF host, whereas during later stages of infection they become inactivated by neutralising antibodies; the same is also believed to be true of exotoxin A (Doring *et al*, 1985). In a study of *P. aeruginosa* from CF patients in good clinical condition, 72% of strains demonstrated proteolytic activity, whereas 60% of isolates from patients in poor clinical condition were

not proteolytic (Luzar & Montie, 1985). The observation that mucoid strains produce less extracellular proteolytic activity than their isogenic non-mucoid revertants (Ohman & Chakrabarty, 1982) also supports the hypothesis that following alginate conversion in the lungs of CF patients, low level production of proteinases may contribute to the unique ability of mucoid *P. aeruginosa* to maintain chronic infections. Experiments by Goldberg & Ohman (1987) indicate transcriptional control over proteinase genes when alginate production is activated.

Pseudomonal alginate has been reported to enhance exolipase activity in a time and concentration dependent manner (Wingender & Winkler, 1984). It remains to be investigated whether lipase contributes as a virulence factor to the persistence of mucoid *P. aeruginosa* in chronic lung infections eg by the destruction of lung tissue or surfactant.

Both exotoxin A, the most toxic exoenzyme of *P. aeruginosa*, and exoenzyme S, which function as distinct ADP ribosyl transferases, are produced by only a minority of isolates from CF patients (Vasil, 1986). Their mode of action is similar to the diphtheria toxin in that both transfer the ADP ribose moiety to elongation factor-2 thereby inhibiting eukaryotic protein synthesis (Fick, 1981). The biosynthesis of exotoxin A, as well as proteases, has been shown to be enhanced in iron-deficient culture (Vasil, 1986) and recent studies demonstrate that this regulation is at the level of transcription (Grant & Vasil, 1986). Use of animal models has indicated the involvement of exotoxin A in histopathological change and respiratory tissue destruction (Baker, 1982; Cash *et al*, 1983), and a possible role in CF. Evidence that exoenzyme S may play a role in lung injury during *P. aeruginosa* infection has been provided by light and electron microscopic characterisation of lung injury after intratracheal instillation of purified exoenzyme S which could reproduce the pathological changes described for *P. aeruginosa* in lung infection (Woods *et al*, 1988).

The haemolysins, phospholipase C, rhamnolipid, and phenazine pigment, have also been postulated to play a role in the pathogenesis of *P. aeruginosa* in CF. In view of the high phospholipid content in the respiratory tract, phospholipase C has been postulated as a potential pseudomonal colonising factor, giving rise to the early phospholipase antibodies observed by Hollsing *et al* (1987). The enzyme may be involved in the degradation of lung surfactant and lung tissue (Liu, 1974).

Kownatski *et al* (1987) found rhamnolipids (heat-stable haemolysin) in sputum from CF patients chronically colonised with *P. aeruginosa*, high concentrations correlating with poor clinical status; the rhamnolipids were absent in non-colonised patients. Rhamnolipids are small detergent like substances which may aid solubilisation of phospholipids, and are also known to inhibit the action of cilia (Hingley *et al*, 1989; Wilson *et al*, 1989).

Bacteria adherent to the mucosa of the CF airway require iron for growth (Fick, 1989). However, in the aerobic environment of the lung, iron is oxidised to Fe^{3+} forming insoluble ferric hydroxide. In addition most of the iron in the respiratory tract is strongly bound by the glycoproteins transferrin, and lactoferrin, the primary iron binding protein on human mucosal surfaces (Fick, 1989; Fick & Hata, 1989).

In situations of iron deprivation *P. aeruginosa* responds by the production of high molecular outer membrane proteins (Brown *et al*, 1984; Cochrane *et al*, 1988a) which may act as receptors for the low molecular weight iron chelators (siderophores) pyochelin and pyoverdine also produced under iron stress conditions (Fick, 1989). In addition the blue, phenolic, phenazine pigment, pyocyanin is also produced to reduce Fe^{3+} to the more soluble Fe^{2+} form, and thus make iron available for bacterial growth (Fick, 1989). The anionic alginate matrix, shown to be stabilised in iron deficient conditions (Boyce & Miller, 1982a) may serve to concentrate iron and siderophores (Shand *et al*, 1989). As

previously indicated pyocyanin may act as a ciliostatic factor (Hingley *et al*, 1989; Wilson *et al*, 1989).

It is a common observation that antibiotic treatment of pulmonary exacerbations due to *P. aeruginosa* in CF patients results in improved lung function and reduction in the levels of the acute phase protein CRP (C-reactive protein) to normal, even when numbers of *P. aeruginosa* present in sputum remains high (Glass *et al*, 1988). This beneficial effect of antibiotics may be due to suppression of pseudomonal virulence and tissue damaging enzymes (Govan *et al*, 1987). In addition, subinhibitory concentrations of antipseudomonal agents have been shown to inhibit adherence of mucoid *P. aeruginosa* to tracheobronchial mucin (Vishwanath *et al*, 1987) and intact hamster tracheal epithelium (Geers & Baker, 1987).

CHAPTER 5

ANTIBODY RESPONSE TO *P. AERUGINOSA* IN CYSTIC FIBROSIS

No specific primary defect in the immunological defence mechanisms of CF patients has been demonstrated (Schiøtz, 1982; Fick, 1989). CF patients exhibit a normal systemic humoral response to various vaccinations (Høiby, 1977) as well as various bacterial, viral, dietary and drug protein antigens (Wallwork *et al*, 1974). In fact the available evidence suggests that antibodies may actually participate in various immunopathological mechanisms which contribute to the pathogenesis of *P. aeruginosa* lung infection in CF (Høiby & Schiøtz, 1982; Høiby *et al*, 1986; Dasgupta *et al*, 1987).

5.1 SERUM AND MUCOSAL ANTIBODIES

Compared with normal children CF patients have normal or increased numbers of circulating B lymphocytes (Høiby & Mathiesen, 1974). Upon antigenic stimulation B lymphocytes are induced to produce immunoglobulin specific for that particular antigen. Immunoglobulins are the major glycoproteins of the humoral immune system and five different classes are recognised: IgG, IgA, IgM, IgD and IgE. In serum, IgG may account for up to 80% of the total immunoglobulin concentration followed by IgA (10-15%), IgM (10%) and less than 1% and 0.01% of IgD and IgE, respectively (Pedersen *et al*, 1989b). Immunoglobulins have a dual function, the Fab region of the molecule constituting the antigen binding site, whilst the Fc region binds to complement and to various host cells, such as neutrophils and macrophages, and mediates immune reactions including opsonophagocytosis and the classical complement pathway. In addition, binding of immunoglobulins to their respective target may mediate immunological protection by neutralization (eg toxins, enzymes) and may include precipitation of soluble antigens, or agglutination of insoluble antigens.

The major mediator of humoral immunity on mucosal surfaces is IgA (Bienenstock & Befus, 1980; Kilian *et al*, 1988) although IgG appears to be the principal opsonin in normal respiratory secretions (Reynolds & Thompson, 1973). IgM antibodies are largely restricted to the bloodstream and are found only in miniscule amounts in the respiratory tract (Kazmierowski *et al*, 1977) probably due to size constraints preventing diffusion across the blood-alveolar barrier (Reynolds, 1989). Secretory IgA is synthesized by local plasma cells as a dimer containing a joining polypeptide J-chain. Another polypeptide, the secretory component, is synthesized in epithelial cells and binds to dimeric IgA forming secretory IgA which is released via the apical membrane to the mucosal surface (Bienenstock & Befus, 1980). Secretory IgA tends to be present in high concentrations in the upper and large airways but diminishes in relative concentration in distal portions of the lung (Reynolds, 1983). This may reflect its importance in the nasopharynx and large airways where it forms part of the mucosal barrier preventing adherence of microorganisms, neutralization of toxins, and inhibition of antigen penetration through the mucosa (Reynolds, 1983; Kilian *et al*, 1988). Because of its less efficient opsonizing potential and uncertain interaction with the complement system (Reynolds & Thompson, 1973; Reynolds, 1983) secretory IgA may be less important in the peripheral airways and alveoli. IgG is the predominant antibody found in these regions (Reynolds, 1983; Bienenstock, 1984; Reynolds, 1989) and is essential to the optimal clearance of *P. aeruginosa* from the distal human airway (Fick & Hata, 1989). Increased levels of IgG in respiratory secretions may be associated with transudation from serum, particularly during respiratory infection (Bienenstock, 1984).

Many studies of the immunoglobulin levels in CF patients have been reported. Schwartz (1966) found that serum IgA and IgG were significantly elevated compared to normal values, whereas IgM and IgD levels were normal; IgA was very high in those patients who subsequently developed pulmonary complica-

tions. In a study of 106 patients, serum IgA and IgG levels were higher in patients who had recently experienced a major episode of chest infection compared to those CF patients without such episodes (Turner *et al*, 1978). Pressler *et al* (1988) reported that a large proportion of both young and old CF patients had increased levels of IgG, whereas IgA and IgM were seen less frequently in patients less than 10 years old. Data from various studies (Højby *et al*, 1975; Matthews *et al*, 1980; Moss & Lewiston, 1980; Cukor *et al*, 1983; Wheeler *et al*, 1984) indicates that the majority of CF patients have normal or elevated levels of immunoglobulins and that *P. aeruginosa* is associated with increased levels of IgA and IgG and a poor clinical condition.

5.2 ANTIBODY RESPONSE TO *P. AERUGINOSA* ANTIGENS

CF patients are capable of mounting an antibody response against cellular and extracellular pseudomonal antigens (Højby, 1977; Pedersen *et al*, 1987). Use of purified *P. aeruginosa* antigens has demonstrated antibodies to the extracellular antigens alkaline, protease, elastase, exotoxin A, phospholipase C and exoenzyme S (Doring & Højby, 1983; Granstrom *et al*, 1984; Doring *et al*, 1985; Hollsing *et al*, 1987; Woods *et al*, 1989). Antibodies to cellular components including alginate (Pier *et al*, 1987), LPS (Fick *et al*, 1986; Jacobson *et al*, 1987; Cochrane *et al*, 1988b; Fomsgaard *et al*, 1988b), outer membrane proteins (Hancock *et al*, 1984); pili (see Pedersen *et al*, 1989b for references) and flagella (Anderson *et al*, 1989) have been demonstrated.

Antibodies against *P. aeruginosa* can be detected by a variety of immunological methods including crossed immunoelectrophoresis (Højby, 1977), radioimmunoassay (Doring & Højby, 1983), enzyme-linked immunosorbent assay (ELISA) (Brett *et al*, 1986b; Pedersen *et al*, 1987) and immunoblotting (Shand *et al*, 1988b). Determination of antibodies against *P. aeruginosa* are useful to diagnose progression from superficial colonisation with *P. aeruginosa* to invasive infection, giving an insight into the mode of pathogenesis of the organism in lung infection (Højby, 1977; Hollsing *et al*, 1987; Pedersen *et al*,

1987).

In a longitudinal study by Doring & Høiby (1983) the immune response increased in nearly all patients after the onset of chronic *P. aeruginosa* infection, showing increases in antibody titres to proteases and increases in the number of precipitins to a water-soluble standard antigen-preparation of *P. aeruginosa*. These results suggested that the action of elastase and alkaline protease are important only at the onset of chronic infection, after which time they are neutralized by specific antibodies. In a related study by Hollsing *et al* (1987) antibodies to a number of *P. aeruginosa* exoproteins were detected, serum antibodies to phospholipase C developing first, at an average of three months after initial colonisation. In contrast Shand *et al* (1989) observed that antibodies to iron regulated membrane proteins occur late in the disease and may reflect a change in the physiological conditions under which *P. aeruginosa* grows in the infected lung.

ELISA studies, with whole cells (Brett *et al*, 1986a & b) or a sonicated mixture of *P. aeruginosa* antigens (Pedersen *et al*, 1987) for the detection of serum IgG antibodies, showed high titres of antibodies in patients chronically colonised by *P. aeruginosa*, antibodies increasing with the duration of infection and correlating with a deterioration in clinical state. In a recent study by Brett *et al* (1990) elevated serum IgA antibodies to *P. aeruginosa* whole cells were particularly associated with patients who were chronically infected by *P. aeruginosa*.

In a longitudinal study of serum antibodies to *Pseudomonas* LPSs during the course of chronic lung infection, a good positive correlation between anti-LPS IgG antibodies and the number of precipitins to *Pseudomonas* sonic extracts was found (Fomsgaard *et al*, 1988b). Earlier studies by Høiby (1977) and Høiby *et al* (1977) showed that high and rapidly increasing numbers of *Pseudomonas* precipitins determined by crossed immuno-electrophoresis, were associated with a poor prognosis including impaired respiratory function, and severe

changes on chest radiographs. A later examination of anti-LPS antibodies in CF patients showed that IgG and IgA anti-LPS antibodies increased significantly at the onset of chronic infection and continued to increase to very high levels in the later stages of infection (Fomsgaard *et al*, 1989). IgM anti-LPS levels also rose at the onset of chronic infection but did not increase further (Fomsgaard *et al*, 1989).

In some patients, antibodies can be detected in serum before sputum bacteriology becomes positive for *P. aeruginosa* (Brett *et al*, 1987; Brett *et al*, 1988; Fomsgaard *et al*, 1988b; Shand *et al*, 1988b; Brett *et al*, 1990). Brett *et al* (1988) found that 73% of CF patients had increased IgG titres up to two years before the first isolation of *P. aeruginosa* from sputum. In a subsequent report, measurement of IgA antibodies was shown to be better than IgG measurement at predicting the reappearance of *P. aeruginosa* after apparent eradication and also gave an earlier indication of the presence of *P. aeruginosa* in the respiratory tract (Brett *et al*, 1990).

Pier *et al* (1987) reported an association between opsonophagocytic killing antibodies in the sera of relatively healthy CF adolescents directed against alginate and a lack of detectable colonisation. Although patients chronically colonised with *P. aeruginosa* demonstrated high titres of serum opsonophagocytic killing antibodies than did the older non-colonised patients, these antibodies were not specific to the mucoid exopolysaccharide, suggesting a role for opsonic killing antibody specific to alginate in resistance to colonisation with *P. aeruginosa* in CF. Other studies have also reported the detection of alginate antibodies in sera from patients with CF (Bryan *et al*, 1983; Pedersen *et al*, 1989a; Pedersen *et al*, 1990). In a recent study Pedersen *et al* (1990) observed that patients responded to *P. aeruginosa* infection with an early IgA and IgG antibody response to alginate although these antibodies did not prevent development of chronic infection, poor lung function correlating significantly with increased levels of IgA and IgG antibodies to alginate. Elevated titres of

anti-alginate antibodies were also found in patients colonised by non-mucoid *P. aeruginosa* indicating that alginate may be produced *in vivo* by apparently non-mucoid organisms.

The presence of *P. aeruginosa* specific antibodies in CF sputa, particularly in those patients chronically colonised by the organism, have been reported by a number of investigators (Schiøtz *et al*, 1979; Przyklenk & Bauernfeind, 1988). In the study of Przyklenk & Bauernfeind (1988) patients chronically colonised by *P. aeruginosa* had significantly higher secretory IgA antibody levels against LPS and elastase compared to non-colonised patients, increases in secretory IgA correlating well with acute exacerbations attributable to *P. aeruginosa*. The ELISA studies of Cochrane *et al* (1988b) demonstrated very low sputum titres of IgG antibodies against LPS. The authors were unable to detect these antibodies in immunoblots of sol phase of sputum, and suggested that LPS-specific antibodies in sputum may be bound to LPS forming immune complexes. Hann & Holsclaw (1976) found that *P. aeruginosa* in CF sputum were coated with IgA, IgG, IgM and complement factor C3 by using a fluorescent technique and that staining was most intense with IgA. In another study bacteria in autopsy lung sections were coated with IgA, IgG and C3, but not IgM (Speert *et al*, 1987).

5.3 IMMUNE DEFECTS IN CYSTIC FIBROSIS

In CF it would appear that despite an apparently competent and often hyper-immune response which protects the host from systemic bacterial invasion, the *P. aeruginosa* specific antibodies are unable to eliminate the organism from the lungs. The implication is therefore that the organism, which may remain localised at the mucosal surface of the respiratory tract for up to 15-20 years (Pedersen *et al*, 1989b), is able to evade the opsonophagocytic host defences.

An important function of IgG is to act as an opsonin for phagocytosis and it has been found that CF serum is able to inhibit the phagocytosis of *P. aeruginosa* by

alveolar macrophages obtained from a number of sources including rabbits and individuals with and without CF (Thomassen *et al*, 1979). However, this inhibition did not affect phagocytosis of *P. aeruginosa* by peripheral monocytes, and neutrophils (Thomassen *et al*, 1982). Fick *et al* (1981) demonstrated a superior affinity of CF derived IgG opsonins to bind to bacteria when compared to other immune opsonins. However, the antibody attachment to alveolar macrophage membrane Fc receptors appeared less avid for the CF specimens and led to the conclusion that CF IgG opsonins acted in an inhibitory fashion (Fick *et al*, 1981). In a study by Eichler *et al* (1989) LPS specific non-opsonic antibodies were detected in some sera from CF patients colonised by *P. aeruginosa* and shown to inhibit the neutrophil oxidative response.

In the lung the presence of secretory IgA antibodies, less able than IgG to mediate opsonization (Reynolds, 1983), may also contribute to the persistence of infection by blocking the bactericidal and opsonizing effect of IgG (Pedersen *et al*, 1989b). The inability of IgG antibodies to kill *P. aeruginosa* may also be caused by the development of blocking antibodies which have been demonstrated in serum (Thomassen & Demko, 1981) and sputum (Schiller & Millard, 1983) from CF patients.

Based on the presence of free J-chain and altered electrophoretic mobility of secretory component isolated from CF sputa, Wallwork & McFarlane (1976) suggested that there may be an abnormality in the synthesis and/or assembly of secretory IgA molecules in patients with CF. However, such reports of a primary deficiency in secretory IgA remain unsubstantiated.

In sputum a defect in the opsonizing ability of IgG as well as IgA, may be caused by the fragmentation of antibody (Fick *et al*, 1984) caused by the proteolytic activity of *P. aeruginosa* elastase (Fick *et al*, 1985) or elastase from polymorphonuclear leucocytes (Doring *et al*, 1986). These enzymes may also inactivate opsonizing complement components such as C3 (Suter *et al*, 1984).

The bacterial elastase is able to cleave IgG in the hinge region (Fick *et al*, 1985), the F(ab)2 and Fc fragments generated closely resembling those detected in the respiratory secretions of CF patients (Fick *et al*, 1984; Bainbridge & Fick, 1989). The polypeptide fragments are unable to promote opsonophagocytosis and are actively inhibitory to pulmonary macrophage and polymorphonuclear leucocyte phagocytic cell uptake of *P. aeruginosa* (Fick *et al*, 1984; Bainbridge & Fick, 1989).

The discovery that immunoglobulins from CF patients have a lower content of galactose and sialic acid compared to non-CF individuals (Margolies & Boat, 1983) may also be due to *P. aeruginosa* induced alteration rather than a primary defect. The organism may produce a glycosidase which secondarily alters the carbohydrate containing hinge region (Fick, 1989). Hypoglycosylated immunoglobulins are cleared less rapidly from the circulation and may contribute to the elevated levels of IgG and immune complexes commonly found in older CF patients (Margolies & Boat, 1983). In addition, alteration in the IgG carbohydrate components may influence intracellular bactericidal processes and serve to protect *P. aeruginosa* within phagocytes (Schoderbek & Fick, 1987). Indeed, it is postulated that it is from this protected intracellular pool that *Pseudomonas* airway infections relapse (Fick, 1989).

5.4 ANTIBODY SUBCLASS AND RELATED IMMUNOLOGICAL IMPLICATIONS IN CYSTIC FIBROSIS

There are four different subclasses of IgG each with characteristic biological functions (Pedersen *et al*, 1989b). IgG subclasses in serum and normal bronchoalveolar fluid have similar subclass proportions in which IgG1 accounts for 65% of total IgG, IgG2 about 30% and IgG3 and IgG4 combined contribute about 5% (Reynolds, 1989). Complement activation is most efficient with IgG1 and IgG3 which also bind well to phagocyte membrane receptors, whereas IgG2 and IgG4 bind complement poorly and are non-opsonic (see Pedersen *et al*, 1989b for references).

The proteolytic activity of *P. aeruginosa* elastase has been shown to be significantly more active on IgG1 and IgG3 (Fick *et al*, 1985), whilst the IgG response to *Pseudomonas* LPS is largely restricted to IgG2 (Fick *et al*, 1986). Indeed, the opsonic index ($\text{IgG1} + \frac{\text{IgG3}}{6} / \text{IgG2} + \text{IgG4}$) has been found to be inverted in CF compared to normal (Fick *et al*, 1986) with a pronounced shift to IgG2 in CF airway secretions demonstrated (Hornick & Fick, 1987). IgG4 levels have been shown to be significantly increased in CF patients and to correlate with disease severity (Shakib *et al*, 1976). The relative surplus of non-opsonic IgG2 and IgG4 antibodies will therefore contribute poorly to phagocytic clearance (Fick *et al*, 1986) and may present a significant impediment to IgG opsonin-mediated phagocytosis of *P. aeruginosa* ie by acting as blocking antibodies as described by Thomassen & Demko (1981). Such a scenario may be particularly relevant in the initial stages of infection before proteases are neutralised by specific antibodies (Doring & Høiby, 1983).

Since the antibody response to polysaccharide antigens such as LPS is predominantly restricted to IgG2 subclass (Siber *et al*, 1980) it would seem that a major determinant in terms of protection is the host's ability to generate a sufficient amount of IgG2 antibody that will be protective (Reynolds, 1989). An IgG2 deficiency in CF patients has been suggested by Moss *et al* (1986b) who found that some infected CF patients had a deficient IgG2 antibody response to *P. aeruginosa* LPS. In contrast however, Albus *et al* (1989) could find no impaired IgG2 response to alginate polysaccharide, and reported significantly elevated levels of all four subclasses to both protein and polysaccharide antigens.

Moss (1987) found that hypergammaglobulinemia associated with CF was mainly due to increased levels of IgG2 and IgG3 and that the striking increases in IgG2 correlated significantly with IgG2 antibodies against LPS. Similarly Pressler *et al* (1988) have found that an elevation of IgG2 and IgG3 correlated

significantly with poor lung function and chronic *P. aeruginosa* infection. It has been postulated that poorly cleared IgG2-bacterial antigen complexes may contribute to immune complex-mediated destruction of the CF airways (Fick, 1989). Current evidence suggests that IgG2 is present in significant quantities in CF immune complexes (Hornick & Fick, 1987).

5.5 IMMUNE COMPLEXES AND IMMUNE MEDIATED TISSUE DAMAGE

Immune complexes, formed by the interaction of antigen and antibody, play an important role in host defences against bacterial infection (Kronborg *et al*, 1989). The immune response and localised inflammatory reactions act together to eliminate the offending pathogen and to neutralize bacterial toxins. If elimination is not accomplished, chronic infection may result which, in turn, may be associated with immunopathological disease. The simultaneous presence of *P. aeruginosa* and the sensitised host's humoral and cellular immune responses suggest that hypersensitivity reactions may contribute to the *Pseudomonas* initiated pathologic-lung-lesion in CF (Fick, 1981; Fick, 1989).

The formation of immune complexes and a subsequent type III hypersensitivity reaction appears to correlate strongly with increasing titres of antibody in serum and sputum from patients with chronic lung infection and is associated with decreasing pulmonary function and poor patient prognosis (Højby & Axelsen, 1973; Moss & Lewiston, 1980; Schiøtz, 1982; Højby & Schiøtz, 1982; Moss *et al*, 1986a; Dasgupta *et al*, 1987). Immune complexes have been detected in CF lung fluids, various tissues and serum by many investigators (Moss & Lewiston, 1980; Doring *et al*, 1984; Dasgupta *et al*, 1987). Pulmonary colonisation by *P. aeruginosa* would result in the formation of immune complexes initially in the lungs, the rate and nature of their further formation depending on the antigenic load, rate of formation of antibodies and the efficiency of the lung's immunologic clearance systems (Dasgupta *et al*, 1987). Circulating immune complexes are believed to be derived as a spill-over effect of immune

complexes from the lungs when the pulmonary clearance mechanisms are saturated or overwhelmed (Schiøtz, 1981; Dasgupta *et al*, 1987). A number of pseudomonal antigens are believed to be present in CF immune complexes including LPS and proteases (Doring *et al*, 1984; Moss *et al*, 1986a).

The biological properties of immune complexes and their ability to participate in type III hypersensitivity reactions is determined by their ability to activate complement and to interact with other host cells (Højby & Schiøtz, 1982). The activation of complement leads to liberation of the complement anaphylatoxins C3a and C5a which in turn attract neutrophils to the lungs, as indicated by the presence of high numbers of these cells in sputum (Højby & Schiøtz, 1982; Suter *et al*, 1984). Accumulation of neutrophils may also be enhanced by the presence of other chemoattractants including *P. aeruginosa* endotoxin and the leucotrienes D and B (Kharazmi *et al*, 1986).

Bronchoalveolar lavage from CF airways reveals the greatly increased numbers of cells and a very abnormal cell differential: 30-90% neutrophils in CF lavage compared to 3% neutrophils in normal individuals (Fick & Reynolds, 1983). The neutrophils moving into the distal airways contribute to the proteolytic damage initiated by *Pseudomonas*, by release of elastase, collagenase and cathepsin G which are able to destroy important structural proteins of the lung and its airways, including elastin, collagen and proteoglycans (Suter *et al*, 1984). The release of other lysosomal enzymes, and of toxic oxygen radicals released during the oxidative burst of activated neutrophils, also contribute substantially to lung damage in CF (Fick, 1981; Højby *et al*, 1986; Kharazmi *et al*, 1987). The report of Auerbach *et al*, (1985) that systemic steroids have a beneficial effect on CF patients indirectly supports the view that immune complex mediated damage is important.

Intermittent immune complex mediated inflammatory exacerbations superimposed on the insidious, chronic lung disease gradually leads to impaired lung

function and eventually the death of the CF patient (Høiby & Schiøtz, 1982). Characteristically the lungs of such patients at autopsy are severely consolidated, containing an abundance of purulent material with high numbers of mucoid *P. aeruginosa* and large numbers of neutrophils (Høiby & Schiøtz, 1982).

AIMS OF THE THESIS

Pulmonary colonisation of patients with CF by *P. aeruginosa* represents a complex, multi-faceted example of bacterial adaptation and pathogenesis. The objective of this thesis was to investigate various aspects of the host-bacterium interaction with a particular focus on the initial colonisation stages with non-mucoid *P. aeruginosa*.

The areas of research covered include:-

- 1) Detection of anti-*P. aeruginosa* antibodies in serum, saliva and sputum from patients with CF. The response to *P. aeruginosa* LPS and flagellar antigens was investigated. The development of a simple ELISA system based on more defined LPS antigenic preparations than those used in previous published studies, and its suitability for the detection of antibodies against all serotypes of *P. aeruginosa* was considered.
- 2) Production of anti-*P. aeruginosa* LPS monoclonal antibodies (Mabs) able to recognise all O-antigenic serotypes of *P. aeruginosa*. The underlying objective of this study was to use the Mabs in a capture ELISA system for the detection of *P. aeruginosa* LPS in CF respiratory secretions as a means of facilitating early identification of respiratory colonisation by non-mucoid *P. aeruginosa*.
- 3) Investigation of the mucinophilic properties of non-mucoid *P. aeruginosa* and their possible contribution to the initial stages of pulmonary colonisation in patients with CF.

MATERIALS AND METHODS

CHAPTER 1

MATERIALS

1.1 PATIENTS AND SPECIMENS

Patients

Specimens of sera, saliva and sputum were obtained from patients with CF attending the paediatric and adult CF clinics in Edinburgh. For the purposes of this thesis, CF patients (age range <1-27 years) were classified on the basis of degree of colonisation with *P. aeruginosa*: a) non-colonised patients ie those patients from whom *P. aeruginosa* had never been isolated, b) intermittently colonised patients, *P. aeruginosa* having been isolated from sputum on one or more occasions but no recognizable pulmonary symptoms attributable to the organism, and c) chronically colonised patients, *P. aeruginosa* having been isolated continuously from their sputum specimens.

Serum, saliva and sputum specimens

Serum obtained from CF patients was aliquoted into micronic tubes (Alpha Laboratories, Eastleigh, Hants) and stored at -20°C until analysis. Control serum was also obtained from blood donors at the Blood Transfusion Centre, Edinburgh. Saliva and sputum specimens obtained from CF patients were stored at -70°C. Prior to storage of sputa, 1.0 ml aliquots in 1.5 ml microcentrifuge tubes (Elkay Products Inc., Shrewsbury, MA, USA), were centrifuged in a microcentrifuge (Micro Centaur, MSE, Crawley, Surrey) at 13,000 g for 15 min and the sol phase retained for analysis.

1.2 BACTERIA

Sputum bacteriology

Bacteriology of routine sputum specimens was performed by Mrs C. J. Doherty (Department of Bacteriology, University Medical School, Edinburgh). Sputa were homogenized in sputalysin (Behring) and following appropriate dilutions, were cultured quantitatively on blood agar, Hartley broth agar (incorporating bacitracin) and *Pseudomonas* Isolation Agar (PIA). These procedures allow the detection of *P. aeruginosa* at concentrations as low as 10^2 CFU/ml⁻¹ sputum. Cultures were identified as *P. aeruginosa* by growth on PIA, production of blue-green pigmentation and where necessary by biochemical tests performed with API 20 E strips (API Laboratories Ltd., Basingstoke, Hants).

Pyocin typing and serotyping of *P. aeruginosa*

Pyocin typing of *P. aeruginosa* strains was by the spotting method described by Fyfe *et al* (1984). Serotyping was performed by the slide agglutination method of Lanyi & Bergan (1978).

Maintenance of bacterial strains

Bacterial strains were maintained at -70°C in 10% (w/v) skim milk (Oxoid L31, Oxoid Ltd., Basingstoke, Hants.). Bacterial suspensions were prepared by emulsifying several single colonies in 1.0 ml volumes of skim milk contained in 2.0 ml plastic screw top vials (Elkay). Four 1.0 ml aliquotes were prepared for each strain. Fresh maintenance cultures were prepared at approximately four monthly intervals.

Bacterial strains

Bacterial strains used in this thesis are shown in Table 1. All other bacterial strains, including clinical isolates of *P. aeruginosa*, *P. cepacia*, *P. maltophilia*, *H. influenzae* and *H. parainfluenzae* are indicated in the relevant section of the Results. All bacterial strain numbers prefixed with JN were isolated from

Table 1. Bacterial Strains.

Bacterial Strains	Description	Source
<u>P. aeruginosa</u> IATS serotype strains O:1-O:16	Originally used in production of PEV (Miler <u>et al</u> , 1977)	Dr I.R. Poxton Dept. of Bacteriology, University Medical School, Edinburgh.
<u>P. aeruginosa</u> Core R-LPS mutants PAC605 PAC611 PAC556 PAC609 PAC608 PAC557	Described by Rowe & Meadow (1983)	
<u>P. aeruginosa</u> PA01	Holloway (1955)	Dr J.R.W. Govan Dept. of Bacteriology, University Medical School, Edinburgh.
PA0578 (mucoid) PA0579 (mucoid)	Fyfe & Govan (1980)	
<u>E. coli</u> K12		
<u>P. aeruginosa</u> M2Fla-	non-flagellate mutant of strain M2 (Stieritz & Holder, 1975)	Dr I.A. Holder Shriners Burn Institute, Cincinnati, Ohio, USA.
M2Rev	motile revertent of M2	
WR-5	non-chemotactic mutant (Craven & Montie, 1981)	
<u>P. aeruginosa</u> J1375 J1385	Friend & Newson (1986)	Dr P. Friend Public Health Laboratory, Truro.
<u>Pseudomonas</u> spp NCIB strains indicated in the relevant section of Results		National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.
<u>P. cepacia</u> J1685 J1695 J1705 J1715 J1725 J1735 J1765 J1775		Communicable Diseases Center, Atlanta, Georgia, USA.

sputum of patients with CF.

1.3 CHEMICALS, MEDIA AND REAGENTS

Chemicals

Unless otherwise indicated all chemicals were Analar grade (BDH Chemicals Ltd., Poole, Dorset), and all solutions were prepared in double distilled water.

Complex media

- a) Nutrient yeast broth (NYB) was Oxoid No.2 supplemented with 0.5% (w/v) yeast extract (Oxoid).
- b) Nutrient agar (NA) was Columbia agar base (Oxoid).
- c) *Pseudomonas* Isolation Agar (PIA) (Difco Laboratories, Detroit, Michigan, USA).
- d) Tryptone Soya Agar (TSA) for pyocin typing studies was Oxoid CM131.
- e) Semi-solid agar for motility studies consisted of:-
 - 0.1% (w/v) tryptone (Difco), 0.3% (w/v) yeast extract (Oxoid),
 - 0.5% (w/v) sodium chloride (BDH), 0.4% (w/v) Bacteriological Agar (Oxoid) and 1 litre of distilled water.
- f) Hartley broth agar (incorporating bacitracin) (Oxoid) - for growth of *H. influenzae* and *H. parainfluenzae*.

All commercial media were prepared by the Bacteriology Media kitchen according to manufacturers' instructions. Media were sterilised by autoclaving at 15 psi for 15 min.

Minimal media

a) MALKA minimal medium used for growth studies was prepared as follows:-

Solution A Na_2HPO_4 (73.4 mg/ml); KH_2PO_4 (32.4 mg/ml)

Solution B $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20.5 mg/ml)

Solution C Sodium succinate (4.0 mg/ml)

Solution D $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.83 mg/ml) in sterile distilled water to which one drop of concentrated hydrochloric acid (HCl) was added.

Solution E $(\text{NH}_4)_2\text{SO}_4$ (50.0 mg/ml)

All chemicals were from BDH. Solutions were prepared with sterile distilled water and were filter sterilised. All solutions except C were stored over chloroform.

To prepare 1 litre of MALKA, 20 ml A, 20 ml B, 20 ml C, 1 ml D and 20 ml E were added to 919 ml sterile distilled water. Variations in the MALKA medium used in the growth experiments are described later.

b) Minimal medium employed for growth of *P. aeruginosa* for chemotaxis assays was made according to Moulton & Montie (1979) and contained (per litre of distilled water): K_2HPO_4 (7.0 g); KH_2PO_4 (3.0 g), $(\text{NH}_4)_2\text{SO}_4$ (1.0 g); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 g) and $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ (2.5 mg) and was supplemented with sodium succinate (0.4% w/v). All chemicals were from BDH.

c) Chemotaxis medium (CM) was made according to Moulton & Montie (1979) and consisted of 0.05 M potassium phosphate buffer at pH7.0 supplemented with 0.005 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (BDH), and 0.1 mM disodium ethylenediamine tetraacetic acid (EDTA) (Sigma Chemical Co., Poole, Dorset).

Amino acids, sugars, mucins and lipopolysaccharides

- a) Amino acids used in chemotaxis assays included alanine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and threonine (all from BDH).
- b) Sugars used in chemotaxis studies and/or growth experiments included L-fucose (Sigma); D-galactose (BDH); D-glucose (BDH); N-acetyl-D-galactosamine (GalNAc) (Sigma); N-acetyl-D-glucosamine (GlcNAc) (BDH); and N-acetylneuraminic acid (NANA) (Koch-Light, Haverhill).

All amino acid and sugar solutions were filter sterilised through 0.2 μ m-pore size membrane filters (Schleicher & Schuell, Dassell, W. Germany).

- c) Mucin preparations. Purified porcine gastric mucin was from BDH. Purified human mucins were obtained from Dr J.K. Sheehan and Dr D.J. Thornton (Department of Biochemistry and Molecular Biology, University of Manchester) and included the following: CF tracheobronchial mucin (gel fraction - Mr 10-12 x 10⁶); CF tracheobronchial mucin (gel fraction - Mr 4-30 x 10⁶) fractionated by gel chromatography into fractions A, B, C and D; CF tracheobronchial mucin (sol fraction - Mr 4 x 10⁶); chronic bronchitic mucin (Mr 20 x 10⁶), normal salivary mucins - large - (approximate Mr 20 x 10⁶) and small (Mr 250,000); asthmatic mucin; CF mucin glycopeptides derived from mucins-sol and gel (Mr 300,000-500,000). The CF mucins were derived from the sputum of a female CF patient, who was 16 years old and infected with *P. aeruginosa*.

All human mucins were supplied and stored as solutions in 4 M guanidinium chloride at 4°C. Prior to use in chemotaxis assays mucins were diluted in CM (100 μ g/ml), added to dialysis tubing (previously boiled for 10 min in a water bath) and dialyzed with stirring against 1 litre of CM at 4°C for 24 h.

d) Lipopolysaccharides. LPS was prepared from *P. aeruginosa* strains as described below. Additional LPS, including preparations from *E. coli* R1 and *Salmonella typhimurium* 1542 were obtained from Dr I.R. Poxton (Department of Bacteriology, University Medical School, Edinburgh). Polyvalent *Pseudomonas* LPS extract vaccine PEV-02, which is comprised of cell wall LPS extracts of sixteen IATS O-serotypes of *P. aeruginosa*, was a gift from Dr P. Hambleton (Porton International). The individual vaccine O-serotype components IATS O:1-O:16 (*Pseudomonas* vaccine antigens PVA) were obtained from Dr G. R. Barclay (Regional Blood Transfusion Centre, Edinburgh).

Antisera

Antisera for O-serotyping corresponded to the International Antigenic Typing Scheme (IATS) (Lanyi & Bergan, 1978) and were donated by Dr T.L. Pitt (Division of Hospital Infection, Central Public Health Laboratory, Colindale). The rabbit anti-flagellar type-a and -b antisera, and the rabbit anti-flagella type-b monoclonal antibody (Mab) were kindly provided by Dr G.H. Shand (Statens Seruminstitut, Copenhagen, Denmark).

1.4 EQUIPMENT

Equipment and apparatus used in the experimental studies is indicated in the relevant section of the text. Manufacturers' addresses are only cited once.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 MEASUREMENT OF BACTERIAL CONCENTRATIONS

a) Viable counts Serial ten-fold dilutions of bacteria were prepared in sterile physiological saline and volumes of 100 μ l were plated on NA plates. Plates were incubated at 37°C for upto 24 h and colonies enumerated using a colony counter (Gallenkamp, Loughborough, Leics.).

b) Measurement of optical density Measurements of absorbance were made at 470 nm (spectrophotometer, Spectronic 20, Bausch & Lomb, supplied by Gallenkamp). An aliquot of suspending medium was retained for use as a blank and as a diluent for optical density (OD) measurements. A standard curve of log bacterial numbers (as determined by viable counts) against OD₄₇₀ was constructed. An optical density of 1.0 at 470 nm indicates a concentration of approximately 10^9 cells of *P. aeruginosa* /ml.

2.2 CULTURE OF BACTERIA

Most bacteria were cultured at 37°C. The *Pseudomonas* spp, excluding *P. aeruginosa* were cultured at 30°C. Bacteria were streaked onto NA or PIA (as appropriate) from skim-milk maintenance cultures. Subculture of bacteria onto fresh plate media was performed no more than seven days after the previous subculture and was performed for up to a maximum of three subcultures. After this time fresh cultures were prepared from maintenance cultures.

Bacteria were inoculated into 10 ml liquid medium to prepare a starter culture, which when required could be inoculated into larger volumes for further

growth. All liquid cultures were incubated in an orbital incubator (Gallenkamp) at 100-120 rpm.

2.3 PREPARATION OF SMOOTH LIPOPOLYSACCHARIDES

Extraction of LPS from *P. aeruginosa* strains expressing the S-LPS phenotype was based on the aqueous phenol method of Westphal & Luderitz (1954). Two litre flasks (ten) containing 1 litre of NYB were inoculated with 2.0 ml of an overnight preculture grown in NYB. The flasks were then incubated in an orbital incubator (120 rpm) at 37°C overnight. Bacterial cells were harvested by centrifugation at 15,000 *g* using a KSB continuous flow system (Dupont UK Ltd., Stevenage, Herts.). Cells were then washed twice in phosphate buffered saline (PBS) with centrifugation (Sorval RC-5B refrigerated superspeed centrifuge, Dupont) at 10,000 *g* for 10 min. The bacterial pellet was frozen at -20°C, freeze dried (Edwards Modylo freeze dryer, Edwards High Vacuum Ltd., Crawley, Surrey) and weighed.

The lyophilized bacterial pellet was finely divided, resuspended to a concentration of 5% w/v in distilled water and heated to 67°C in a water bath. Meanwhile 90% w/w aqueous phenol was prepared by dissolving 90 g of phenol (BDH) in 10 ml of distilled water at 45°C and then making the volume up to 100 ml with distilled water. A volume of phenol solution equal to that of the bacterial suspension was heated to 67°C in a water bath. The prewarmed bacterial suspension and phenol solution were mixed and stirred at 67°C for 15 min. The mixture was transferred to 50 ml centrifuge tubes and cooled in an ice bath to allow separation of the phenol and aqueous phases. The tubes were then centrifuged at 10,000 *g* for 15 min to complete separation of the phases. The upper (aqueous) phase containing the LPS was carefully removed and the extraction procedure repeated on the lower phenol phase. The two aqueous phases were pooled, transferred to dialysis tubing (previously washed and boiled for 10 min in distilled water) and dialysed against running tapwater overnight, until the smell of phenol was no longer detectable. The solution was

centrifuged twice for 15 min at 10,000 g to remove any insoluble deposit. The dialysed extract was then concentrated by rotary evaporation (Buchi Rotavapor-RE111, Switzerland) to approximately one-fifth of its original volume. After this the solution was ultracentrifuged (Sorval ultracentrifuge - OTD65B, Dupont) at 100,000 g for 3 h. The gelatinous pellet obtained was resuspended in distilled water with the aid of a syringe fitted with 23-gauge needle and recentrifuged. The final pellet was then suspended in a small volume of distilled water (pyrogen free), freeze dried and weighed. The LPS was stored at -20°C until required.

2.4 PREPARATION OF ROUGH LIPOPOLYSACCHARIDES

The aqueous phenol, chloroform, petroleum ether (PCP) method of Galanos *et al* (1969) incorporating the diethyl ether precipitation of LPS described by Qureshi *et al* (1982), was used to prepare LPS from *P. aeruginosa* expressing the R-LPS phenotype. Bacteria were cultured and lyophilized as described for S-LPS preparations.

The extraction solvent (PCP) consisted of 90% phenol (BDH - see above), chloroform (BDH) and petroleum spirit (boiling point 40°- 60°C) (BDH) in the proportions 2:5:8 by volume. Dried bacteria were resuspended in PCP at 25% w/v, stirred for 2 min below 20°C and then centrifuged at 10,000 g for 15 min. The supernate was filtered through Whatman No. 1 filter paper into a round-bottomed flask. The centrifuged pellet was re-extracted by the same procedure and then the filtered supernates pooled. The supernate was rotary evaporated to remove the petroleum spirit and chloroform.

In order to precipitate the LPS (Qureshi *et al*, 1982) six volumes of diethyl ether/acetone (BDH) in the ratio of 1:5 by volume, were added to one volume of the phenol solution. After leaving to stand for at least 1 h the LPS was sedimented by centrifugation at 5,000 g for 10 min. The centrifuge tubes were drained and the pellet washed three times with diethyl ether/acetone, the

pellet being recovered by centrifugation each time. The final pellet was dried under vacuum until the smell of ether/acetone was no longer detectable. The dried pellet of LPS was resuspended in 5 ml of distilled water with the aid of a syringe and 23-gauge needle and recovered by ultracentrifugation at 100,000 *g* for 4 h. The LPS was then taken up in a minimum amount of distilled water, lyophilized and stored at -20°C until used.

2.5 PROTEINASE K DIGESTION OF BACTERIA FOR THE PREPARATION OF LIPOPOLYSACCHARIDES

The proteinase K digestion of bacteria (Hitchcock & Brown, 1983), suitable for both R- and S-LPS types, was used to prepare LPS for analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Bacteria, from a 10 ml overnight culture, were harvested by centrifugation and washed twice in PBS. The bacteria were resuspended in PBS to give an absorbance between 0.5 and 0.6 at 525 nm as measured on a spectrophotometer. 1.5 ml of bacteria suspension was transferred to an Eppendorf tube (Elkay) and the bacteria sedimented in a microcentrifuge at 1,000 *g* for 3 min. The pellet was resuspended in 50 µl of single strength PAGE sample buffer (see page 85) and heated at 100°C for 10 min. Once cooled 10 µl of sample buffer containing 25 µg of proteinase K (Sigma protease Type X1) (2.5 mg/ml in sample buffer) was added followed by incubation in a 60°C water bath for 60 min. Samples were stored at -20°C.

2.6 PREPARATION OF FLAGELLA

For flagellar antigen preparation, 2 litre flasks containing 1 litre of NYB were inoculated with 2.0 ml of an overnight preculture grown in NYB. The flasks were incubated in an orbital incubator (120 rpm) at 37°C for overnight culture. Bacterial cultures were then harvested by centrifugation at 10,000 *g* for 15 min at 4°C and washed twice in PBS. Each bacterial pellet was suspended in 20 ml of PBS and blended with a commercial blender for 2 min. Bacterial cells were removed by up to twelve cycles of centrifugation (10,000 *g* for 10 min at 4°C).

Removal of cells was monitored at intervals by microscopy and culture of the supernatant. The cell free supernatant was ultracentrifuged at 100,000 g for 2 h at 4°C, and the resultant pellet washed twice in distilled water. The pellet was then resuspended in 0.5 ml distilled water, dialysed overnight against distilled water at 4°C and stored at -20°C until required. The amount of protein in each sample was determined by the method of Lowry *et al* (1951).

Flagella were classified into *P. aeruginosa* flagellar types-a or -b based on their mobility in polyacrylamide gels (12% acrylamide), and by ELISA and immunoblot analysis with rabbit anti-flagellar type-a and -b antisera.

2.7 ELECTRON MICROSCOPY OF FLAGELLA

Flagellar preparations were examined by electron microscopy and were prepared in two ways:

a) Negative-staining with phosphotungstic acid (PTA) Formvar-coated 400 mesh copper grids were floated onto a drop (approximately 5 µl of flagellar sample, previously diluted 1:10 in distilled water. Excess moisture on the grids was removed by lightly blotting with filter paper (Whatman No. 1). Grids were then touched onto a drop of 2% w/v PTA before drying with filter paper and allowing to dry fully in a desiccator.

b) Shadowing with platinum Grids were prepared with flagella as above. Prior to shadowing grids were allowed to dry fully in a desiccator for at least 30 min. Grids were then shadowed with platinum in an Edwards vacuum coater model 306 (Edwards High Vacuum).

All grids were viewed with a Hitachi 12A electron microscope at 75 KV.

2.8 PREPARATION OF SAMPLES FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Sample buffer (pH6.8) contained 0.0625 M Tris (BDH) at pH6.8 in which 2.0% w/v sodium dodecyl sulphate (SDS) (BDH Specially Pure), 10% v/v glycerol (BDH), 1% v/v 2-mercaptoethanol (BDH) and 0.001% bromophenol blue (BDH) were present. Double strength sample buffer (pH6.8) was made as above except concentrations of all constituents were doubled.

a) Protein samples The protein concentration of flagellar preparations was determined by the method of Lowry *et al* (1951) and adjusted to 1.0 mg/ml with distilled water. Samples were then mixed with an equal volume of double strength sample buffer and were denatured by heating at 100°C for 10 min in a water bath.

b) Lipopolysaccharide samples Proteinase K digests were prepared as described above and added to gels at 10 µl per track. Samples of extracted LPS (1.0 mg/ml), were mixed with an equal volume of single strength buffer and heated at 100°C for 10 min before electrophoretic analysis. Samples were added at 10 µg LPS per track.

2.9 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE was performed with the buffer system of Laemmli (1970). PAGE of proteinase K samples for subsequent immunoblotting was carried out with SDS free gel buffers (Pyle & Schill, 1985). The following buffers and solutions were used in PAGE:

- a) Electrode buffer (pH8.3) consisted of 0.025 M Tris (BDH), 0.192 M glycine (BDH Chromatographically Homogeneous) and 0.1% w/v SDS (BDH).
- b) Separating gel buffer (double strength, pH8.8) consisted of 0.75 M Tris-HCl at pH8.8 to which 0.2% w/v SDS was added. (SDS omitted for SDS-free buffer).
- c) Stacking gel buffer (double strength, pH6.8) consisted of 0.25 M Tris-HCl at

pH6.8 to which 0.2% w/v SDS was added (SDS omitted for SDS-free buffer).

- d) Acrylamide stock solution (40% w/v) contained 100 g acrylamide (BDH Electrophoresis Grade) and 2.7 g methylene bis acrylamide (BDH Electrophoresis Grade) and made up to 250 ml with distilled water.

The separating gel was prepared as described in Table 2, with deaeration under vacuum prior to addition of TEMED (NNN'N'- tetramethyl-1,2-diaminoethane (BDH Electran) and ammonium persulphate (BDH). The gel solution was poured between clean (with methanol) glass plates (160 mm x 125 mm x 1.5 mm) of the slab gel electrophoresis equipment. The gel was overlaid with water saturated butan-2-ol (BDH) and allowed to set. After removal of butan-2-ol the stacking gel, prepared in a similar manner (Table 2), was poured onto the separating gel. A comb was inserted and the gel allowed to set. On removing the comb the gel was fitted into an electrophoresis tank (Jencons Scientific Ltd., Leighton Buzzard, Beds.) and the electrode buffer added.

Samples, prepared as described above, were loaded into the wells of the stacking gel. Samples were electrophoresed through the stacking gel at a constant 60 V and through the separating gel at a constant 150 V until the dye front had run 80 mm. After electrophoresis samples were analysed by staining or by immunoblotting after transfer to nitrocellulose.

2.10 SILVER STAINING OF POLYACRYLAMIDE GELS FOR LIPOPOLYSACCHARIDE

An adaptation of the methods of Tsai & Frasch (1982) and Hitchcock & Brown (1983) was used to visualise LPS in polyacrylamide gels. The following reagents and solutions were used in the silver staining procedure:

- a) Fixative consisted of 7% v/v acetic acid (BDH General Purpose) and propan-2-ol (BDH General Purpose).

Table 2. Preparation of polyacrylamide gels.

Reagent	Volume (ml) to give acrylamide concentration of:		
	12% Separating gel	14% Separating gel	4% Stacking gel
Distilled water	5.2	3.45	3.5
Separating buffer	17.5	17.5	-
Stacking buffer	-	-	5.0
Acrylamide solution (40% w/v)	10.5	12.25	1.0
TEMED	0.05	0.05	0.02
Ammonium persulphate (15mg/ml)	1.75	1.75	0.5

- b) Oxidising solution contained 1.05 g periodic acid (BDH General Purpose) in 150 ml of distilled water to which 4 ml of fixative was added.
- c) Ammoniacal silver nitrate solution was made up by mixing 1.4 ml ammonia solution (BDH General Purpose) with 21 ml of 0.36% w/v sodium hydroxide (BDH) and the slow addition of 4 ml silver nitrate (BDH) accompanied by vigorous agitation. Volume was made up to 100 ml with distilled water.
- d) Developing solution consisted of 0.019% v/v formaldehyde (BDH) solution containing 0.005% w/v citric acid (BDH).

Post-electrophoresis the gel was placed in fixative and left overnight at room temperature. Fixative was poured off and the gel oxidised for 5 min with a freshly prepared solution of periodic acid. The gel was then washed in at least four changes of 200 ml distilled water over 4 h. Silver staining was carried out by addition of fresh ammoniacal silver nitrate solution. After staining for 10 min the gel was washed with at least four changes of distilled water (200 ml) over 40 min. LPS was visualised by the addition of 200 ml of the freshly made developing solution. Once the desired staining intensity had been obtained (5-10 min) the gel was washed repeatedly in large volumes of distilled water. The gel was stored in the dark. (All the above reaction steps were shaken).

2.11 COOMASSIE BLUE STAINING OF POLYACRYLAMIDE GELS FOR PROTEIN

The Coomassie blue stain described by Hancock & Poxton (1988) was used. The solutions used were made up in distilled water and included:

- a) Solution 1 - 25% v/v propan-2-ol (BDH General Purpose), 10% v/v acetic acid (BDH General Purpose) and 0.05% w/v Coomassie brilliant blue R-250 (BDH).
- b) Solution 2 - 10% v/v propan-2-ol, 10% v/v acetic acid and 0.005% w/v Coomassie blue.
- c) Solution 3 - 10% v/v acetic acid and 0.0025% w/v Coomassie blue.

- d) Solution 4 - 40% v/v methanol (BDH General Purpose) and 10% v/v acetic acid.
- e) Solution 5 - 10% v/v acetic acid.

The gel was placed in solution 1 overnight and then sequentially through solutions 2-5, each for 45-60 min at room temperature with gentle shaking throughout .

2.12 IMMUNOBLOTTING

Bacterial cell components separated in PAGE were transferred to nitrocellulose (NIC) paper (pore size 0.2 μm) (Schleicher & Schuell, Dassel, W. Germany) for immunochemical analysis. A modification of the method of Towbin *et al* (1979) was used.

The following reagents and solutions were used:

- a) Electrode transfer buffer (pH8.3) - contained 12 g Tris (BDH General Purpose), 57.68 g glycine (BDH Chromatographically Homogeneous) in 4 litres distilled water and 1 litre methanol (BDH General Purpose).
- b) Tris buffered saline (pH7.5) (TBS) - contained 4.84 g Tris (BDH) 58.48 g sodium chloride (BDH) and 2 litres distilled water.
- c) Tween-Tris-buffered saline (pH7.5) (TTBS) - as for TBS containing 0.025% v/v Tween 20 (Sigma).
- d) Blocking solution - as for TBS containing 3% w/v gelatin (Bio-Rad EIA Purity Grade, Bio-Rad Laboratories Ltd., Watford, Herts.).
- e) Antibody/conjugate diluent - as for TBS containing 1% w/v gelatin (Bio-Rad).
- f) Horse radish peroxidase (HRP) colour solution. The development solution was made up fresh immediately before use. 30 mg HRP colour reagent (Bio-Rad EIA Purity Grade) was dissolved in 10 ml methanol (BDH) and added to 50 ml TBS containing 50 μl hydrogen peroxide (BDH General Purpose).

Electrophoretic transfer of antigens from polyacrylamide gels to nitrocellulose

The gel was removed from the PAGE apparatus and placed in the ScotchbriteTM pad cassette of the blotting apparatus (made in-house by Mr J. Duffus, Dept. of Bacteriology, University Medical School, Edinburgh). The gel was then covered with a sheet of NIC presoaked in transfer buffer. The cassette was then closed sandwiching the gel and NIC paper between the two Scotchbrite pads. The assembly was then placed into the immunoblotting tank containing transfer buffer, ensuring that the gel was placed towards the cathode and the NIC towards the anode. A constant current of 40 mA was applied overnight at 4°C.

Visualisation of antibody-antigen reactions

After transfer the NIC was removed and washed in TBS for 10 min before placing in blocking solution for 30-45 min. Once blocked the NIC was transferred into antibody buffer containing an appropriate dilution of sample (serum, saliva, sputum or Mab) and incubated for 3 h at room temperature. The NIC was then rinsed briefly in distilled water and washed for two 10 min periods in TTBS, following which it was incubated with HRP-conjugated secondary antibody suitably diluted in antibody diluent. The type of conjugate used depended upon the particular investigation (see Results) and included the following: anti-human IgA; anti-human IgG; anti-mouse Ig; anti-rabbit IgG (all from ICN Biomedicals Limited, High Wycombe, Bucks.). The NIC was treated for 60 min with the secondary antibody followed by rinsing in distilled water and washing in TTBS (as above). The binding of antibody to separated antigenic determinants was visualised by addition of HRP colour solution to the NIC. The colour was allowed to develop for up to 30 min and then stopped by washing in several changes of distilled water. The blot was dried and stored in the dark. (The above processing stages involved gentle shaking throughout).

2.13 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Polystyrene microplates (Immulon M129A) (Dynatech Billingshurst, Sussex) and polystyrene microwell strips (Polysorb F8) (Nunc, Inter Med, Kamstrup, Roskilde, Denmark) were used in the ELISA experiments. Frame-modules were used to hold strips to form a 'plate'. Mini-sorb tubes (Nunc) were used for making antigen dilutions. The following diluents and buffers were used during the various ELISA procedures:

- a) Coating buffer (pH9.6) consisted of 0.05 M carbonate/bicarbonate buffer (6.2 g/litre $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ and 4.2 g/litre NaHCO_3) plus 0.05% w/v sodium azide (Sigma).
- b) Post-coat buffer consisted of PBS (pH7.2) containing 5% v/v bovine serum albumin (BSA) (ICN Biomedicals) and 0.05% w/v sodium azide.
- c) Wash buffer consisted of PBS (pH7.2) containing 0.05% v/v Tween-20 (Sigma) and 0.05% w/v sodium azide.
- d) Dilution buffer consisted of PBS (pH7.2) containing 0.05% v/v Tween-20, 4% w/v polyethylene glycol 6,000 (Sigma) and 0.05% w/v sodium azide.

ELISA coating procedures

- a) Coating with LPS-polymyxin complex (Scott & Barclay, 1987)

Stock solutions of LPS and polymyxin were made up in distilled water and were sonicated (Microson, Ultrasonic Cell Disruptor, Heat Systems-Ultrasonics Inc, NY, USA) for 30 s prior to mixing. Complexes were formed by adding polymyxin B sulphate (Sigma) at a concentration of 0.2 mM with LPS at a concentration of 0.1 mM and then sonicating for another 30 s. The mixture was then transferred to cellulose dialysis tubing with a 2,000 molecular weight cut off (Spectrum Medical Industries Inc. Los Angeles, USA) and dialysed overnight at 4°C against distilled water (containing 0.05% w/v sodium azide) to remove unbound polymyxin. Complexes were stored in mini-sorb tubes at -20°C until required. Molarity of *P. aeruginosa* R- and S-LPS was calculated from the estimated molecular weights of LPS as determined by Morrison &

Jacobs (1976) as shown in Table 3.

To coat microwell strips or microtitre plates, complex was diluted 1:50 in coating buffer and added at 100 µl per well. Strips and plates were coated overnight at room temperature and washed four times with wash buffer (Microwash 2, Skatron, Traby, Lier, Norway). Wells were post-coated with post-coat buffer at 100 µl per well overnight at room temperature. After being washed four times with wash buffer, strips and plates were rinsed in distilled water and stored at -20°C until used.

Cocktails of different LPS-polymyxin complexes (see Results) were constituted by mixing equal volumes of the relevant complexes. The coating procedure was as described above.

b) Coating with *Pseudomonas* extract vaccine (PEV-02) and individual vaccine serotype components (PVA)

PEV-02 was diluted 1:500 in coating buffer giving a final carbohydrate concentration of 1 µg/ml. The individual vaccine O-serotype components (stock solutions 0.1 mg/ml in distilled water) were diluted 1:100 in coating buffer giving a final concentration of 1 µg/ml. Both were added to wells at 100 µl per well. Incubation, washing and post-coating was carried out as described for LPS- polymyxin coating.

c) Coating with whole bacteria

Bacteria were grown overnight in NYB, harvested and washed twice with PBS. Cells were resuspended to a density of 10^7 cells/ml (determined spectrophotometrically) in coating buffer and added at 100 µl per well. Plates were then centrifuged at 1,365 g for 5 min to sediment bacteria to the wells. Following overnight coating at room temperature the procedures were as described for LPS-polymyxin coating.

Table 3. Molecular Weight of LPS.

LPS TYPE	MOLECULAR WEIGHT	<u>P. AERUGINOSA</u> EQUIVALENT
Wild type (smooth)	15,000	Serotype strains
Ra and Rb type	4,500	PAC557 PAC608 PAC556 PAC611
Rc type	4,150	PAC605
Rd and Re type	3,100	

d) Coating with *P. aeruginosa* flagellar antigens

Flagellar antigen preparations were diluted in coating buffer to a concentration of 1 µg/ml and added to microwell strips at 100 µl per well. The coating procedure was as described above.

ELISA experimental procedures

The coated microwell strips/plates were used in a number of ELISA studies which will be described individually for ease of reference:

a) Detection of serum IgG anti-*P. aeruginosa* LPS antibodies

Plates coated with PEV-02, R-LPS/polymyxin complexes, and whole cells, and strips coated with the above, plus vaccine components PVA O:1-O:16 were used in these studies. Sera were diluted 1:200 in dilution buffer and added at 100 µl per well in triplicate. Plates were incubated at 37°C for 90 min before washing four times with wash buffer. Urease-conjugated anti-human IgG (Sera Lab Ltd., Crawley Down, Sussex) was diluted 1:500 in diluent, added at 100 µl per well and plates incubated for a further 90 min at 37°C. Plates were washed four times and rinsed with distilled water before substrate (Sera Lab) at 100 µl per well was added. Plates were incubated for 30 min at room temperature and reactions stopped by adding 1% w/v thimerosal in distilled water (20 µl per well). The OD of wells was read at 590 nm on an automated microplate reader (Titertek Multiskan/MC, Flow Laboratories Ltd., Irvine). Final results were expressed after subtraction of the OD of negative control wells (coated only with BSA - post coat) for each serum.

b) Detection of salivary and sputum IgA and IgG anti-*P. aeruginosa* LPS antibodies

Plates coated with PEV-02 and R-LPS/polymyxin samples were used in these studies. Dilutions of saliva and sputum were made in dilution buffer and added at 100 µl/well in triplicate. Plates were incubated at 37°C for 90 min before

washing four times with wash buffer. HRP-conjugated anti-human IgA and IgG (both ICN) were diluted 1:1000 and added at 100 µl per well and plates incubated for a further 90 min at 37°C. Plates were washed four times and rinsed with distilled water before substrate at 100 µl per well was added. Substrate consisted of 0.1 M sodium acetate (BDH)/citrate (Sigma) buffer (pH6.0) containing 1 mg/ml 3,3',5,5'-tetramethyl-benzidine (ICN) made up in dimethylsulphoxide (BDH) and 0.015% v/v hydrogen peroxide (BDH General Purpose). Plates were incubated for 60 min at room temperature and reactions stopped by adding 2 M sulphuric acid at 25 µl per well. The OD of wells was read at 450 nm on a microplate reader. Final results were expressed after subtraction of the OD of negative control wells (as above).

c) Detection of serum, salivary and sputum IgA and IgG anti-*P. aeruginosa* flagellar IgA and IgG antibodies

As for b) above.

d) Screening of mouse sera and Mabs antibodies for anti-*P. aeruginosa* LPS activity

Sera from mouse tail bleeds were diluted 1:100 in diluent. Supernatant from hybrids and clones were screened for Mab at a dilution of 1:5. Screenings included microwell strips coated with LPS/polymyxin complexes, PEV-02, PVA serotype vaccine components, and whole cell preparations. 'Antibody' samples were added at 100 µl per well and plates incubated at 37°C for 90 mins before washing four times with wash buffer. Urease-conjugated anti-mouse Ig, IgG or IgM (all Sera Lab) (used as appropriate - see Results) were diluted 1:500, added at 100 µl per well, and plates incubated for a further 90 min at 37°C. Plates were washed and rinsed before substrate (Sera Lab) at 100 µl per well was added. Plates were incubated for 60 min at room temperature and reactions, if required, stopped by thimerosal (as for a) above). OD of wells was read at 590 nm on a microplate reader and results expressed after subtraction of negative controls (as above).

2.14 ABSORPTION OF CLINICAL SPECIMENS

a) Absorption of sera with whole cells

Sera from CF patients were subjected to a series of absorptions with *P. aeruginosa* whole cells. Overnight cultures of bacteria grown in NYB were harvested by centrifugation (4,600 g for 15 min), washed twice in PBS and resuspended to a density of 10^7 cells/ml. Bacterial suspension (1 ml) was added to a 1.5 ml Eppendorf tube and bacteria harvested at 1,365 g for 5 min using a microcentrifuge. After removal of supernatant, bacteria were resuspended in serum diluted 1:200 in dilution buffer, incubated for 15 min at room temperature, and recentrifuged. The supernatant was then added to another pellet of cells and the process repeated. This step was repeated at least three times for each absorbing cell type to ensure maximal absorption of antibodies by bacteria. Serum anti-pseudomonal LPS IgG antibodies in absorbed and unabsorbed sera were assayed by ELISA.

b) Absorption of clinical specimens with extracted LPS

To avoid immunological detection of anti-LPS antibodies in the flagella experiments, due to any contaminating LPS (Poxton *et al*, 1985), specimens were preabsorbed with LPS. Sera, saliva and sputa (100 µl) were mixed with an equal volume of extracted LPS at 1 mg/ml. (The LPS was from the strain used to prepare the corresponding flagella). Samples were incubated for 60 min at 37°C followed by incubation overnight at 4°C. Afterwards samples were suitably diluted prior to use in ELISA and immunoblot studies. Removal of LPS antibodies was confirmed by ELISA against the relevant LPS.

2.15 PRODUCTION OF ANTI-*P. AERUGINOSA* LPS MONOCLONAL ANTIBODIES

a) Preparation of cells for mice vaccinations

Bacteria were cultured overnight in NYB, harvested by centrifugation (4,600 g for 15 min) washed twice with PBS and resuspended in PBS (pH7.2) to a density of 10^8 cells/ml. Cell suspensions were then dispensed in 10 ml aliquots into

sterile universal bottles and were heat-killed by heating at 100°C for 5 min. Bacterial culture of the heat-killed preparations was used to ensure total bacterial killing. Samples were frozen at -20°C and used as required.

b) Mice vaccinations and Mab production

All animal manipulations were performed by Mr J. Verth (Animal House, University Medical School, Edinburgh) and production of Mabs was by Dr L.R. Micklem (Dept. Surgery, University Medical School, Edinburgh). **BALB/c** mice were immunised with 0.1 ml of the heat-killed cell preparations (equivalent to 10^7 cells/ml) as follows: day 1, 2, 3, 8, 9, 10 with PAC608; day 22, 23, 24 with PAC605. Blood from the mice was taken at: pre- inoculation day 1, and day 20, 31 and 38. The bloods were allowed to clot overnight at 4°C and then centrifuged at 13,000 g for 30 s in a microcentrifuge. The supernatant serum was collected and stored at -20°C.

The nature of the antibody response to *P. aeruginosa* LPS was measured by ELISA. A mouse showing a strong, cross-reactive antibody response to *P. aeruginosa* LPS was selected (see Results). The mouse was boosted intravenously with 0.1 ml heat-killed cells of PAC608 and killed 3 days later to obtain spleen cells. Mouse spleen cells were fused with cells of the NS-1 (NS-1/1-Ag4-1) plasmacytoma cell line. Resulting hybrids were screened for anti-*P. aeruginosa* LPS antibodies by ELISA. Antibody positive hybrids were cloned by limiting dilution and were also screened in ELISA (see Results). Clones producing Mabs of particular interest were grown in bulk (1 litre) for the concentration and partial purification of the Mabs.

c) Concentration of Mabs from bulk growths

Culture supernatant from bulk growth (1 litre) of Mab producing clones was centrifuged at 10,000 g for 10 min to remove cellular debris. The supernatant containing Mabs was concentrated 20-fold by a Minitan tangential-flow system (Millipore, Bedford, MA, USA) employing Minitan pore filters (Millipore) with

a molecular weight cut-off of 30,000. The system was purged with copious amounts of pyrogen free distilled water and 0.1 M sodium hydroxide (BDH) and primed with a small quantity of RPMI (Gibco, Paisley, Renfrewshire) between each concentration. Activity of the Mabs was checked by ELISA.

d) Ammonium sulphate precipitation of immunoglobulins

The method used was a modification of that described by Hardy (1986). Immunoglobulin was precipitated by the addition of crystalline ammonium sulphate (Sigma Grade III) to the concentrated solution containing Mab to give 50% saturation (equivalent to a final concentration of 0.313 g/ml). Thus 3.13 g of ammonium sulphate was added slowly, with stirring to 10 ml of the Mab preparation. The ammonium sulphate was allowed to dissolve and the precipitate was stirred for 2 h at room temperature. The precipitate was harvested by centrifugation at 10,000 g for 60 min at 4°C and the pellet resuspended in 10 ml of 0.1 M NaHCO₃ (BDH). The solution was reprecipitated, recentrifuged and resuspended as above. The ammonium sulphate was removed by dialysis for 20 h at 4°C with stirring, against at least three changes of 0.1 M NaHCO₃ buffer containing 0.05% w/v sodium azide (Sigma). Activity of the Mabs was checked by ELISA.

2.16 AGAROSE GEL ELECTROPHORESIS

Mabs prepared by ammonium sulphate precipitation were analysed by agarose gel electrophoresis based on a modification of the method described by Carlstrom & Johansson (1983). The solutions and reagents required included:

- a) Tris-glycine-vernal buffer (TGV) (pH8.6). 25.75 g of barbitone and 16 g of sodium barbitone (both BDH) were dissolved in 1 litre of distilled water and added to a further 1 litre of water containing 17 g of glycine (BDH Chromatographically Homogeneous) and 13.75 g of Tris (BDH). The mixture was stirred, 37.2 g of disodium EDTA (Sigma) added and the mixture made up to a final volume of 5 litres.
- b) Agarose gel. A 1% w/v solution of agarose (Bio-Rad standard low- *Mr*) in

TGV was prepared with the aid of a boiling water bath. After allowing to cool 60⁰-70⁰C, 22.2 ml was poured onto the hydrophilic surface of a sheet of GelbondTM (Bio-Rad) (125 mm x 100 mm) supported on a glass plate on a levelling table (Bio-Rad). The gel was allowed to set at room temperature for 5 min and then placed in a fridge for 30 min to harden.

- c) Picric acid fixative. A warmed (60⁰C), filtered solution of 1.4% w/v picric acid (BDH) in water was mixed with glacial acetic acid (BDH) in a ratio of 5:1 (v/v).
- d) Staining and washing solution for protein. Methanol, acetic acid (both BDH General Purpose) and distilled water were mixed in 9:2:9 ratio (v/v). The dye free solvent was used for the washing solution. Coomassie brilliant blue R-250 (BDH) was added at 0.2% w/v for the staining solution.

The area of the gel to be used for placement of the applicator foil was lightly blotted with Whatman No. 1 filter paper to remove surface moisture. The applicator (containing a series of regularly arranged slots for diffusion of samples into the gel) was then placed on the gel. Samples (2 μ l), previously diluted 1:2 in 0.1% w/v bromophenol blue in TGV, were applied to slits using microcapillaries. Samples were left for 5 min after the last application in order to allow diffusion into the gel. After diffusion excess sample was removed from the applicator which was then removed. The gel was placed in the electrophoresis cell tank (Model 1415, Bio-Rad) and the wicks (five layers of Whatman No. 1 filter paper) were put in place to serve as contacts between each end of the gel and the respective TGV buffer reservoirs. A glass plate was then placed on top to prevent the wicks becoming detached from the gel surface. Cooled water at 10⁰C from a temperature control unit (Model RTE-5DD, Jencons Scientific) was circulated through the gel tank at a flow rate of 1 litre per min. The gel was run at a constant 300 V until the dye front had travelled 55 min.

The gel was placed in picric acid fixing solution for 15 min and then dried by

covering with six sheets of filter paper and pressing for 5 min. Drying was completed by placing in a 60°C oven for 15 min. The thin gel film was briefly rinsed in distilled water and stained for 10 min. After staining the gel was washed twice with dye free solvent until a colourless background was obtained.

2.17 BIOTINYLATION OF MONOCLONAL ANTIBODIES

Biotinylation of Mabs was based on the procedure described by Guesdon *et al* (1979). Amino hexanoyl-biotin-N-hydroxysuccinimide ester (AH- BNHS) (Zymed Laboratories Inc., San Francisco, California, USA) was used to covalently bind biotin to the Mabs. The protein concentration of Mab preparations was determined by the method of Lowry *et al* (1951). Calculations of quantities of reagents were based on a value of 90 free amino groups per gamma globulin molecule as reported by Habeeb (1966). Various molar ratios (1:1, 2:1, 4:1) of AH-BNHS and free amino groups of gamma globulin Mab molecules were reacted together to determine the optimal binding ratio. To react a 1:1 molar ratio of AH-BNHS with the free amino groups of Mab, 27.5 µl of AH-BNHS (10 mg/ml dissolved in dimethylformamide BDH) was added to 1 ml of Mab (1 mg/ml in 0.1 M NaHCO₃). Reactions were performed in Reactovials TM (Pierce Chemical Co., Rockford, Illinois, USA) and were vigorously stirred for 2 h at room temperature. The reaction mixture was dialysed for 24 h at 4°C with stirring, against several changes of PBS (pH7.2) containing 0.05% w/v sodium azide (Sigma). After dialysis an equal volume of glycerol (Sigma) was added and the biotinylated Mabs (bio-Mabs) stored at -20°C. The bio-Mabs were analysed by ELISA.

2.18 ISOTYPING OF IMMUNOGLOBULINS

Mouse Mabs were isotyped by a blotting detection kit for mouse antibodies (RPN29) (Amersham International plc, Amersham, Bucks.).

2.19 STREPTAVIDIN-BIOTIN ELISA SYSTEMS

ELISA studies of bio-Mabs included comparison of immunoassay detection systems involving different streptavidin-labelled enzymes as well as an amplification system incorporating streptavidin-biotin-enzyme complexes. Buffers, reagents and methodologies used in these ELISA studies were as previously described unless otherwise indicated.

- a) Enzyme labelled reagents. Some or all of the following enzyme-labelled reagents were used in the various experiments: i) urease conjugated streptavidin (Sera Lab) diluted 1:200; ii) alkaline phosphatase conjugated streptavidin (Zymed) diluted 1:1000; iii) biotinylated alkaline phosphatase-streptavidin (BAPS) complex made up by the addition of 10 μ l of biotinylated alkaline phosphatase (1 mg/ml) (Zymed) and 20 μ l of streptavidin (1 mg/ml) (Zymed) to 5 ml diluent. The dilution buffer described previously (see page 91) was used for i) and ii). PBS buffer (pH7.2) containing 0.05% v/v Tween 20 (Sigma) was used for iii).
- b) Substrates i) Urease substrate (Sera Lab) was used for the urease-conjugated streptavidin; ii) substrate for the alkaline phosphatase labelled reagents was prepared by dissolving one p-nitrophenyl phosphate tablet (Sigma 104 phosphatase substrate tablets) per 5 ml of substrate dilution buffer (pH9.8 - containing 0.05 M sodium carbonate/bicarbonate, 0.002 M magnesium chloride).

Bio-Mabs were serially diluted (or as appropriate) in diluent and added to coated microwell strips at 100 μ l per well. In some of the investigations (checkerboard titrations) strips were coated with a series of different antigen concentrations. Plates were incubated for 90 min at 37°C and then washed. Enzyme-labelled reagents were added at 100 μ l per well and plates incubated for a further 90 min prior to washing and then rinsing with distilled water. The relevant substrate was added (100 μ l per well) and plates incubated for 60 min at room temperature prior to reading the OD - 590 nm for urease and 405 nm for alkaline phosphatase.

2.20 MONOCLONAL ANTIBODY COMPETITION STUDIES

An ELISA competition assay was devised to compare the antigen binding sites of different Mabs. Buffers, reagents and methodologies were as previously described. A dilution series of unlabelled Mab was prepared in dilution buffer and added to coated microwell strips at 100 μ l per well. Following incubation (90 min at 37°C) bio-Mab (the same or different to the unlabelled Mab) serially diluted in diluent, was added on top of the unlabelled Mab at 100 μ l per well ie in checkerboard fashion. Plates were shaken for 15-30 s to mix Mabs, incubated (90 min at 37°C), and then washed prior to addition of 100 μ l per well streptavidin alkaline phosphatase (1:1000). Following incubation (90 min at 37°C) plates were washed, rinsed and substrate added at 100 μ l per well. After incubation (60 min at room temperature) the OD was read at 405 nm.

A standard curve was established by titrating one Mab against itself ie the same Mab used for both the label and competitor. The capacity of other unlabelled Mabs to inhibit the binding of the labelled Mab was titrated. The results were plotted and the concentrations necessary to achieve 50% inhibition of binding were compared.

2.21 CAPTURE ELISA

A capture ELISA was developed for the detection of *P. aeruginosa* LPS in solution. Solutions, reagents and basic methodologies were as previously described.

The wells of microwell strips were coated with Mab diluted, as appropriate (see Results), in coating buffer. Plates were coated overnight at room temperature and then washed, post-coated and subsequently stored as previously described. A series of dilutions of *P. aeruginosa* LPS, whole-cells and heat-killed cells were made in dilution buffer and added at 100 μ l per well. Plates were

incubated at 37°C for 2 h before washing. Bio-Mab, diluted as appropriate (see Results), was added at 100 µl per well and plates incubated at 37°C for 90 min. After washing, BAPS complex (see page 101) was added at 100 µl per well and plates incubated for 30 min at 37°C. Plates were then washed and alkaline phosphatase substrate added at 100 µl per well. Following incubation (60 min at room temperature) the OD of wells was read at 405 nm.

The above method was used for detection of LPS in saliva and sputum specimens. Prior to use in the assay samples were deproteinised by heating (100°C for 5 min) as recommended by Doskeland & Berdal (1980). Samples were then diluted 1:2 in diluent and added at 100 µl per well to Mab coated wells. The remainder of the assay was as described above.

2.22 BACTERIAL CHEMOTAXIS ASSAY

Bacterial chemotaxis was measured by the capillary tube assay described by Adler (1973) incorporating the minimal medium and chemotaxis medium (CM) described by Moulton & Montie (1979). One millilitre of an overnight preculture grown in minimal medium was inoculated into 9 ml of minimal medium and incubated at 37°C for 3 h in an orbital incubator at 120 rpm. The exponentially growing cells were harvested by centrifugation at 4,600 g for 15 min and washed twice with 10 ml of CM before suspension to a density of 2×10^8 CFU (OD 0.25 at 590 nm).

The capillary tube assay was set up and performed as follows: A small chamber was formed by placing a cover slip on a U-shaped piece of melting-point capillary tubing bonded to a microscope slide. Prior to use in the assays, the chambers, supported and enclosed in petri-dishes, were prewarmed by incubation at 37°C for at least 30 min. The chambers were then filled with 0.4 ml of bacterial suspension in CM.

Capillaries (0.25 mm internal diameter, 45 mm in length and sealed at one end by flaming) (Phase Separations Ltd., Deeside, Clwyd) were filled with potential chemoattractants dissolved in CM. Control capillaries containing CM alone were used in all experiments as a measure of background motility. The open end of a capillary was inserted into the centre of a chamber containing the bacterial suspension. After incubation for 30 min (unless otherwise indicated) the capillaries were removed, their sealed ends carefully broken with a diamond scribe, and the contents emptied into 1 ml of sterile saline with the aid of a micropipette bulb. The contents were serially diluted in saline and plate counts on NA made.

The accumulation of bacteria in each of two capillaries containing potential chemoattractant was measured as CFU per capillary and a mean value determined. A similar measurement was made from capillaries in which no chemoattractant was present. The ratio of these CFU values (the relative response) was then determined ie the ratio of the accumulation in attractant capillaries to that in control capillaries. The ratio normalizes for experimental or day to day variations. A meaningful chemotactic response as described by Moulton & Montie (1979) is a relative response value of >2.0 .

Some of the mucin preparations were treated with protease (Sigma Type XXV pronase) prior to use in chemotaxis assays. Pronase made up at 1 mg/ml in CM was added to the mucin preparation to a final concentration of 100 $\mu\text{g/ml}$. Samples were incubated at 37°C for 24 h in a water bath prior to use in chemotaxis assays.

2.23 MOTILITY ASSAYS

a) Motility inhibition assay. Motility inhibition studies were conducted by the chemotaxis capillary assay described above. The anti-*P. aeruginosa* flagellar (type-b) Mab was added to the bacterial suspension to a final dilution of 1:40.

The bacterial/Mab solution was incubated at 37°C for 30 min prior to use in the chemotaxis assay. Motility of cells with and without addition of the anti-flagellar Mab was checked by phase contrast microscopy. Duplicate assays were performed and the relative chemotactic responses obtained from test and control assays compared.

b) Motility assay in semi-solid agar. Motility medium was prepared as described on page 76. Bacterial strains were cultured on PIA and five colonies for each strain were stab inoculated into the centre of the motility medium. For each assay triplicate plates per strain were incubated at 37°C and examined for colony spreading. Colony diameter was measured after 24 h.

2.24 ADHERENCE STUDIES OF *P. AERUGINOSA* TO MUCIN

Two novel methods were developed during the course of this thesis to examine adherence of *P. aeruginosa* to mucin.

Electron microscopy

Visualisation of the mucin-bacterium association by transmission electron microscopy incorporated a modification of a surfactant monolayer technique described by Sheehan *et al* 1986 for the study of mucin architecture.

A log phase culture of bacteria grown in 10 ml NYB was centrifuged at 4,600 *g* for 15 min and washed twice in 1% w/v ammonium acetate (Sigma) in distilled water. The pellet was then gently resuspended in 15 ml of mucin solution (0.5 µg/ml in 50 mM magnesium acetate) (BDH), and the mixture was transferred to a plastic petri dish and incubated for 30 min at 37°C. To prepare mucin monolayers a small amount of fine graphite powder (BDH) was sprinkled on the surface. A mucin monolayer was obtained by touching the surface of the mucin solution with the tip of a pipette containing 1 µl of 1% w/v benzyldimethylalkyl-ammonium chloride (BAC) (Sigma) in distilled water.

The surfactant nature of the BAC propels the carbon particles to the side of the dish, leaving a fine surface monolayer of mucin in BAC. After 15 min, carbon-coated formvar 400-mesh electron microscope grids were touched to the surface of the monolayer, stained for 1-2 s in a solution of uranylacetate (1 mM in 95% v/v ethanol BDH) and washed for 1-2 s in ethanol. The grids were then allowed to dry in a desiccator before being subjected to unidirectional shadowing with platinum at a grazing angle of 7-12°. Transmission electron microscopy was performed with a 12A Hitachi microscope at 75 KV. Grids were examined for mucin-bacterial interactions.

Microtitre ELISA

Many of the materials, reagents and solutions used in the development of this adherence assay were as described previously (see page 91).

Mucins were diluted in coating buffer and added to wells at 100 µl per well. In preliminary experiments mucin concentrations, doubly diluted in coating buffer from 1.6 µg/ml to 200 µg/ml were investigated. A coating concentration of 100 µg/ml was shown to give optimal sensitivity, yielding maximal difference in OD between mucin coated wells and background controls. Wells were coated overnight at room temperature and washed four times with wash buffer. Non-specific binding sites were blocked by post-coating wells overnight at room temperature with post-coat buffer. (Wells coated only with post-coat were used as background controls).

An overnight culture of bacteria was harvested by centrifugation (4,600 g for 15 min) and washed twice in PBS. Cells were then resuspended in PBS containing 1% v/v sterile BSA (Organon-Technika, Cambridge, Cambs.) and 0.05% v/v Tween 20 (Sigma). In preliminary experiments bacterial concentrations 10^5 - 10^9 cells/ml were investigated in the adherence assays. A bacterial concentration of 10^7 cells/ml showed maximal measureable binding to mucin coated wells and minimal non-specific binding to background control wells.

Each individual experiment was performed in duplicate with three wells per determination. Bacterial cells were added to wells at 100 μ l per well in triplicate and incubated at 37°C for 60 min. Wells were then washed eight times with wash buffer. A Mab, cross-reactive with all serotypes of *P. aeruginosa*, diluted 1:500 in dilution buffer, was added at 100 μ l per well. The optimal dilution of Mab was predetermined in initial experiments. After incubation at 37°C for 90 min, wells were washed four times with wash buffer. Urease-conjugated anti-mouse Ig (Sera Lab) diluted 1:500 was added at 100 μ l per well. After incubation (90 min at 37°C) wells were washed four times and rinsed with distilled water, before substrate (Sera Lab) at 100 μ l per well was added. Plates were incubated for 60 min at room temperature before OD of wells was read at 590 nm on a microplate reader. Results for each adherence determination were expressed as the mean OD from triplicate wells after subtraction of the OD of negative background controls. The mean from duplicate experiments was then calculated to give the final result.

2.25 EFFECT OF MUCIN SUGARS ON ADHERENCE

The effect of the mucin sugars L-fucose, D-galactose, GalNAc, GlcNAc and NANA on *P. aeruginosa* adherence to mucins was also investigated by the above assay. Bacterial suspensions (10^7 cells/ml) were prepared in the PBS suspension buffer as described above and were mixed with an equal amount of suspension buffer (controls) or 200 mM sugar solutions in the same buffer, ie equal concentrations of bacteria in buffer or 100 mM sugar solutions were prepared. Solutions of NANA prepared in the PBS buffer had a pH of ca. pH2.5 which is lethal for the bacteria. Therefore, bacterial suspensions were prepared in solutions of NANA dissolved in Tris-HCl buffer and buffered to pH7.2. The bacterial-sugar suspensions were mixed, incubated at room temperature for 60 min, and then added in triplicate at 100 μ l per well. The remainder of the assay was as above. The effect of sugars on adherence of

P. aeruginosa to mucins was expressed as a percentage of the OD obtained without added sugar.

RESULTS

CHAPTER 1

DETECTION OF ANTIBODIES TO *P. AERUGINOSA* LPS

1.1 SERUM ANTI-LPS ANTIBODIES DETECTED BY ELISA

The aim of this study was to produce an ELISA system based on defined antigenic preparations, suitable for the routine detection of an antibody response against all serotypes of *P. aeruginosa*. In the ELISA system developed, two different coating antigens were employed for detection of anti-*P. aeruginosa* LPS antibodies: i) polyvalent *Pseudomonas* smooth LPS vaccine (PEV-02) composed of cell wall LPS extracts of 16 international serotypes of *P. aeruginosa*; ii) LPS from the rough mutant *P. aeruginosa* PAC605, deficient in the ability to produce O-antigenic LPS.

Preliminary experiments were performed to determine the optimal coating concentration of PEV-02, and PAC605 LPS complexed with polymyxin. The results of checkerboard type titrations indicated that PEV-02 at 1 µg/ml or PAC605 LPS at 10 ng/ml and serum from a CF patient chronically colonised by *P. aeruginosa* produced strong readable absorbance values (OD>1.0) within a reasonable substrate reaction time (30 min). In all subsequent experiments a serum sample was classed as positive for anti-*P. aeruginosa* LPS antibodies if the OD value was >0.1 after subtraction of background control.

Serum IgG anti-*P. aeruginosa* LPS antibodies in CF patients variously colonised by *P. aeruginosa* are shown in Figure 4. A range of elevated levels (OD>0.1) of anti-pseudomonal IgG antibodies directed to both PEV-02 S-LPS and PAC605 R-LPS were demonstrated in sera from twenty patients with CF who were chronically colonised by *P. aeruginosa*. Anti-pseudomonal IgG antibody levels in sera from seven intermittently colonised CF patients were

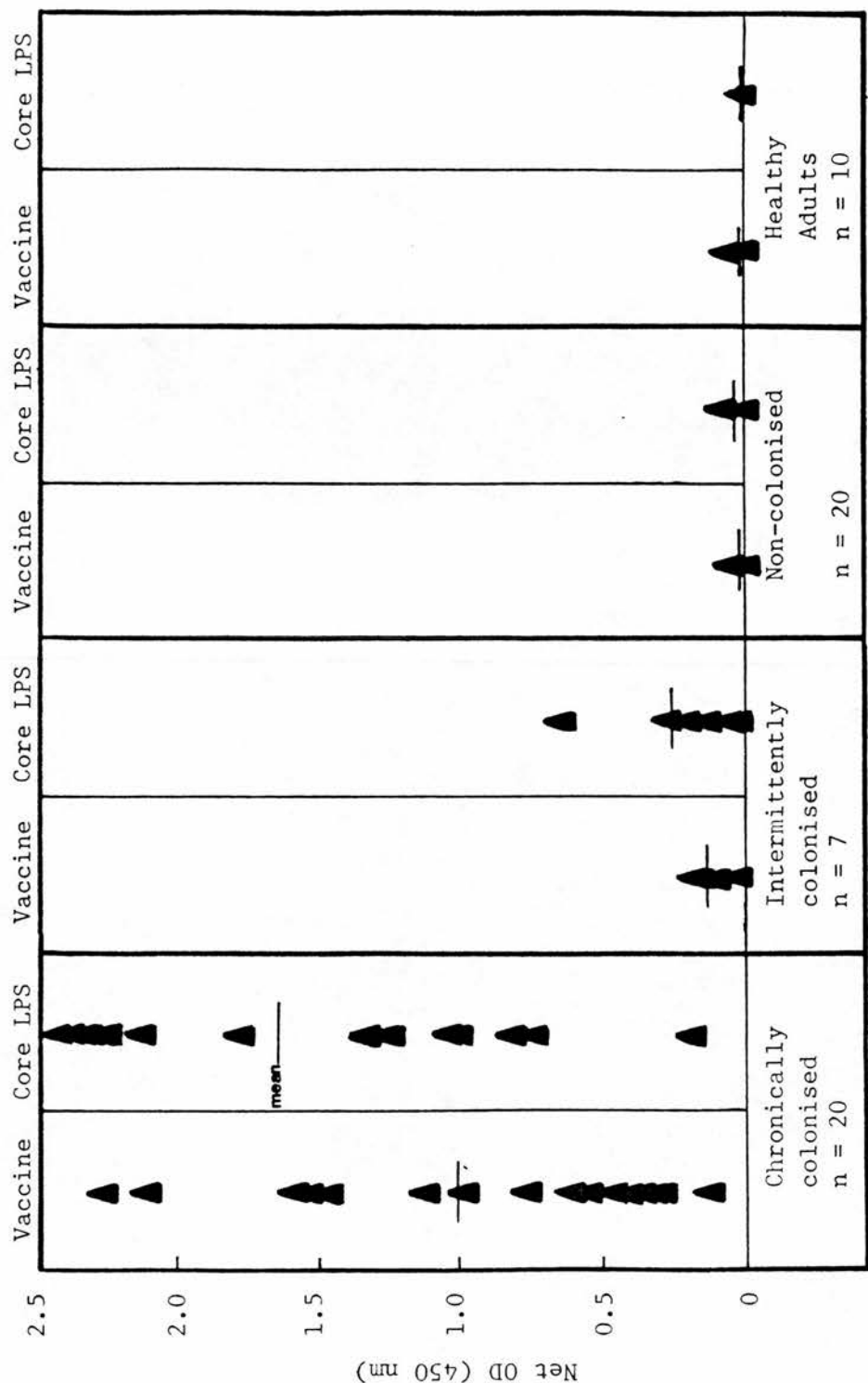


Figure 4. Serum IgG anti-*P. aeruginosa* LPS antibodies in 47 CF patients and 10 non-CF individuals measured by ELISA with either heterogenous polyvalent S-LPS vaccine (PEV-02) or core R-LPS (PAC605) as coating antigens: a total of 20 CF patients chronically colonised with *P. aeruginosa*, 7 intermittently colonised, 20 non-colonised CF patients and 10 healthy adults are included.

substantially reduced compared to the chronically colonised group although a detectable ($OD > 0.1$) response to PEV-02 or PAC605 LPS was observed in some of these patients. Raised anti-pseudomonal IgG antibody levels were not found in sera from twenty non-*P. aeruginosa* colonised CF patients or in ten healthy, non-CF controls, with the exception of one CF patient ($OD = 0.124$) and one non-CF patient ($OD = 0.119$).

Statistical analysis of the results by the Student *t* test revealed: i) the difference in absorbance readings between chronically colonised CF patients, and those intermittently or non-colonised, or healthy control individuals, was very highly significant ($P < 0.001$); ii) the difference in absorbance readings between intermittently colonised CF patients and both non-colonised patients and healthy controls was very highly significant ($P < 0.001$) for PEV-02, and highly significant ($P < 0.01$) for PAC605 core LPS; iii) there was no significant difference in absorbance readings between non-*P. aeruginosa* colonised CF patients and healthy controls.

Reproducibility of the assay was checked with twelve serum samples, four from each of the three groups of CF patients ie chronically colonised with *P. aeruginosa*; intermittently colonised and non- colonised. The results of duplicate assays performed on five different occasions gave an intraplate variation of 4%, an interplate variation of 6.5% and a day to day variation of 12%.

1.2 LONGITUDINAL STUDIES OF SERUM IgG ANTI-LPS ANTIBODIES

The ELISA system described above was used to follow the serum IgG anti-*P. aeruginosa* LPS antibody response in three CF patients who were initially intermittently or non-colonised by *P. aeruginosa* (Figure 5) Low, but detectable ($OD > 0.1$) levels of IgG antibodies directed to *P. aeruginosa* LPS were found in the two patients initially intermittently colonised by the organism.

Antibody levels to both PEV-02 S-LPS and PAC605 core R-LPS increased at the same time as *P. aeruginosa* was cultured consistently (three consecutive occasions) from sputum specimens obtained from each patient. Antibody levels to the two LPS antigens continued to increase and then plateau during the ongoing infection.

1.3 SERUM ANTIBODY RESPONSE TO INDIVIDUAL LPS SEROTYPES OF *P. AERUGINOSA*

The serum IgG antibody response to individual LPS serotypes of *P. aeruginosa* was measured by ELISA with the S-LPS O-serotype vaccine components, PVA O:1-O:16. Serum from four patients chronically colonised with *P. aeruginosa* produced a positive reaction with most of the sixteen individual serotype vaccine components (Figure 6). Given the heterogeneity of antibody response, a pronounced antibody response to any one particular serotype was not evident. Indeed, a pronounced response to the S-LPS of the three most commonly isolated *P. aeruginosa* serotypes (O:1, O:6, O:11) found in the Edinburgh area was not evident, with equally strong responses occurring with some of the less common serotypes.

In contrast, sera from non-colonised or intermittently colonised CF patients produced barely detectable or undetectable antibody responses to the individual serotype components (Figure 7).

1.4 SPUTUM AND SALIVA ANTI-LPS ANTIBODIES DETECTED BY ELISA

Anti-*P. aeruginosa* LPS IgA and IgG antibodies in sputum and saliva were also measured by ELISA with PEV-02 S-LPS and PAC605 R-LPS as coating antigens.

A range of levels of anti-pseudomonal IgA antibodies directed to both PEV-02 and PAC605 LPS were demonstrated in sputa from patients with CF (Figure

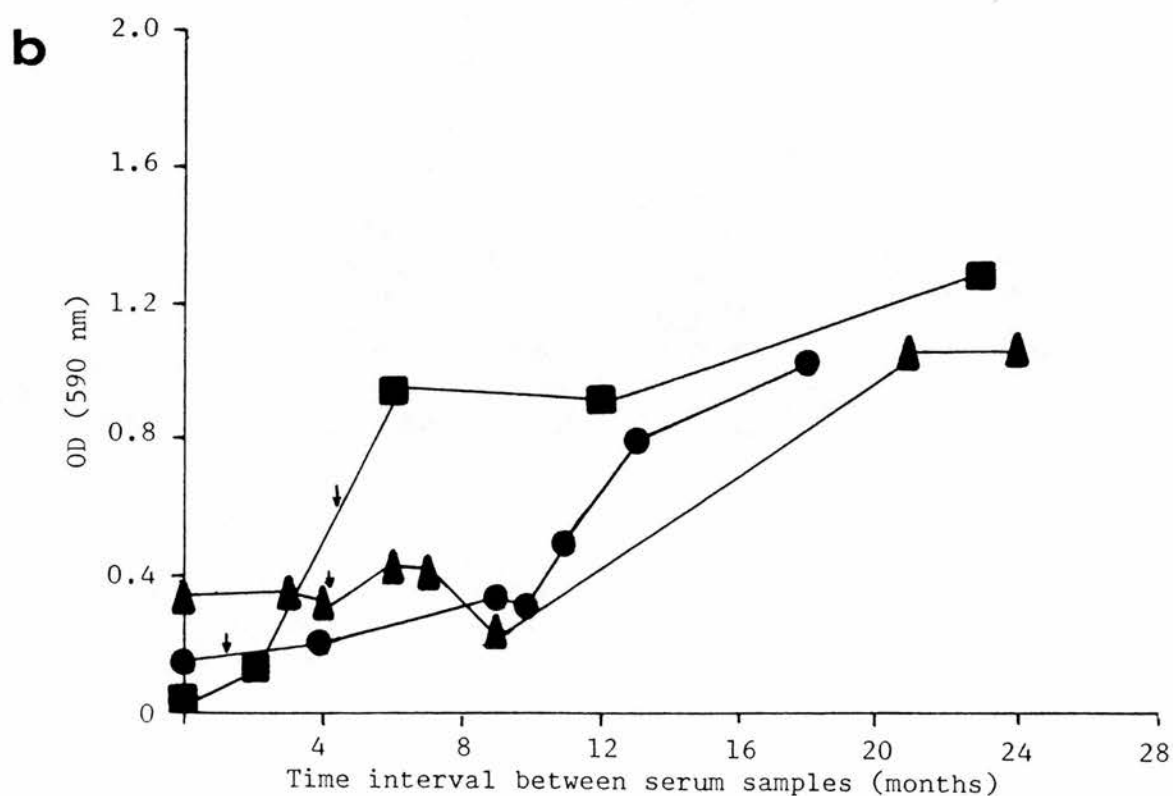
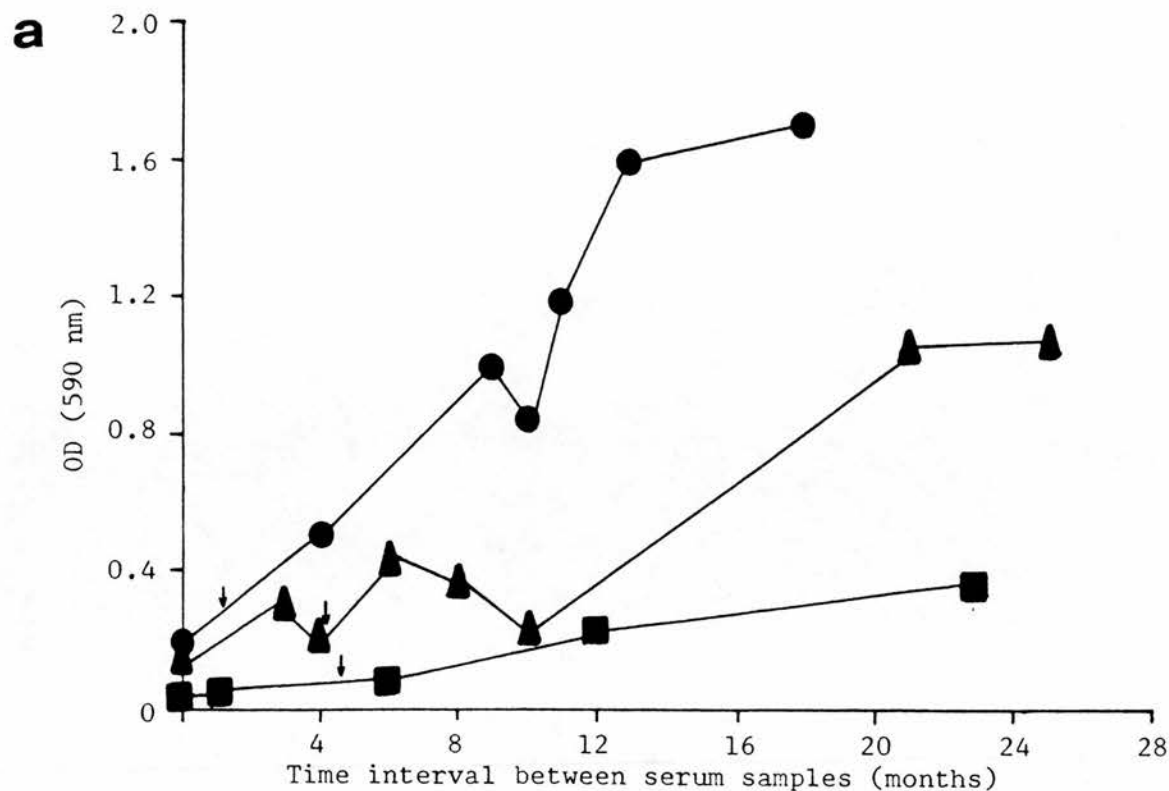


Figure 5. Longitudinal study of serum IgG anti-*P. aeruginosa* LPS antibodies in 3 CF patients measured by ELISA with either a) heterogenous polyvalent S-LPS vaccine (PEV-02) or b) core R-LPS (PAC605) as coating antigens. The arrows indicate the time when *P. aeruginosa* started to be consistently isolated from sputum obtained from each respective patient.

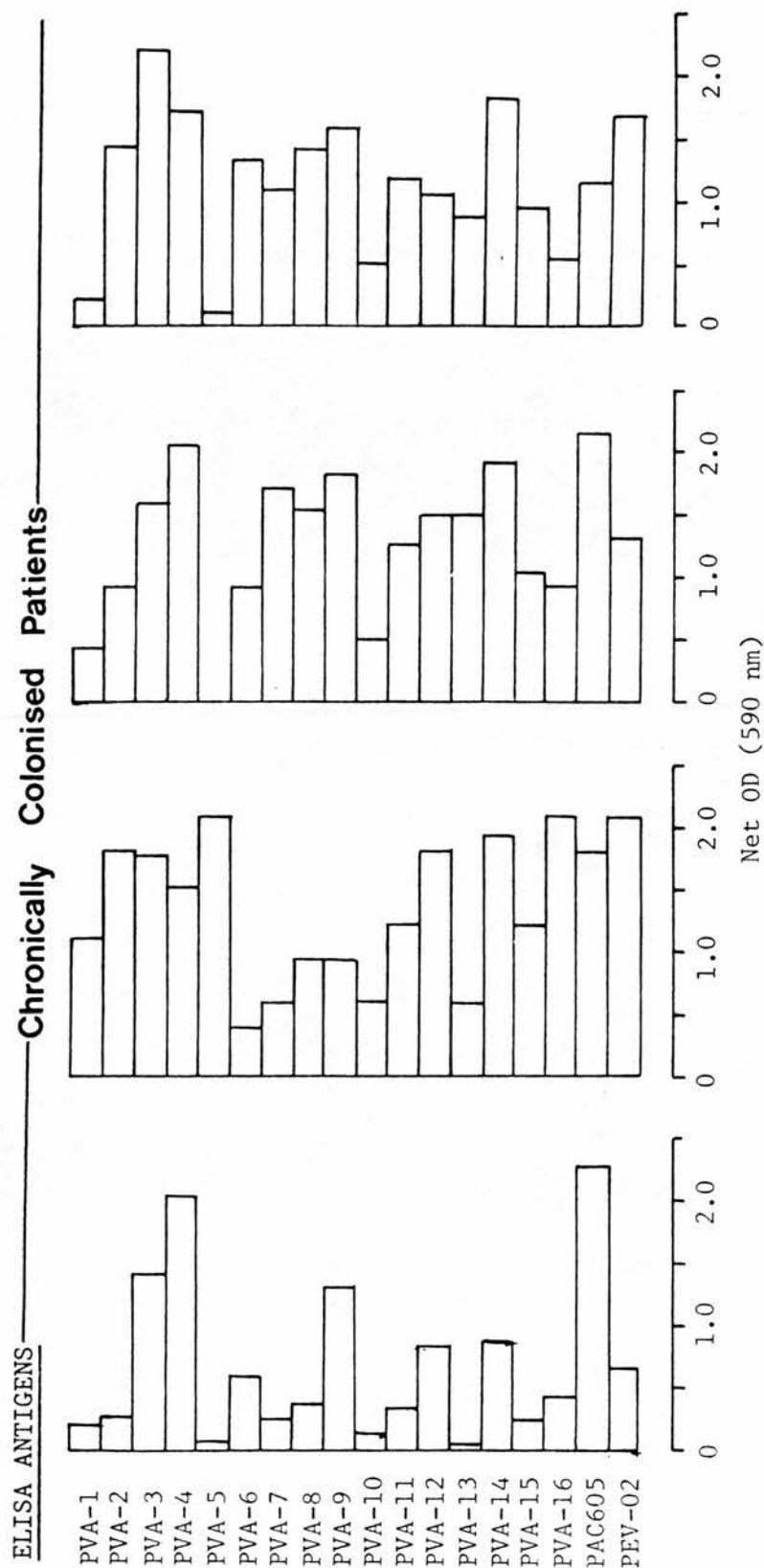


Figure 6. Serum IgG anti-*P. aeruginosa* LPS antibodies in 4 CF patients chronically colonised with *P. aeruginosa* measured by ELISA with individual S-LPS O-serotype vaccine components (PVA-1 to PVA-16), core LPS (PAC605) and polyvalent vaccine (PEV-02).

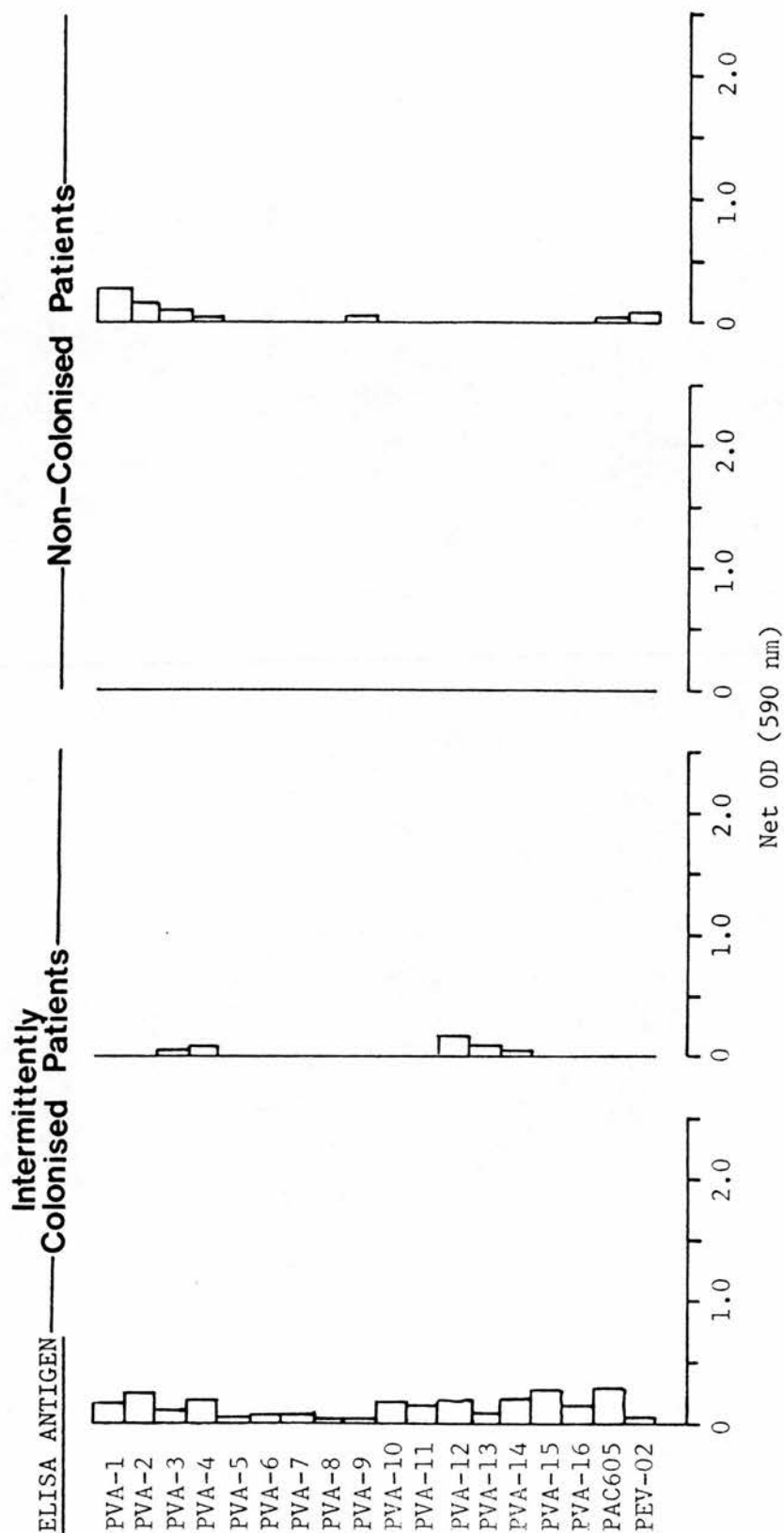


Figure 7. Serum IgG anti-*P. aeruginosa* LPS antibodies in CF patients intermittently or non-colonised with *P. aeruginosa* measured by ELISA with individual S-LPS O-serotype vaccine components (PVA-1 to PVA- 16), core R-LPS (PAC605) and polyvalent vaccine (PEV-02).

8). Patients chronically colonised with *P. aeruginosa* tended to show a greater anti-pseudomonal LPS IgA response than the intermittently, or non-colonised patients, although detectable levels of anti-LPS IgA antibodies were observed in some of both these groups of patients. In the patients chronically colonised by *P. aeruginosa* there tended to be a greater IgA antibody response towards the R-LPS of PAC605 compared to the S-LPS of PEV-02. Statistical analysis by the Student *t* test revealed that this difference was highly significant ($P < 0.01$).

Further statistical analysis of the ELISA results for sputum anti-LPS IgA antibodies showed: i) the difference in OD values between patients chronically colonised with *P. aeruginosa* and non-*P. aeruginosa* colonised patients was significant ($P < 0.02$) for PEV-02 S-LPS and very highly significant ($P < 0.001$) for PAC605 R-LPS; ii) the difference between chronically colonised patients and intermittently colonised patients was not significant for antibodies directed to PEV-02 S-LPS, but was significant ($P < 0.02$) for IgA antibodies directed to PAC605 R-LPS; iii) there was no significant difference in IgA antibody levels between intermittently and non-*P. aeruginosa* colonised CF patients.

In contrast to the anti-pseudomonal LPS IgA antibodies found in sputum, anti-pseudomonal LPS IgG antibodies were not detected (data not shown).

Anti-pseudomonal LPS IgA antibodies were also detected in saliva samples from patients with CF (Figure 9). Patients chronically colonised with *P. aeruginosa* again showed a tendency towards a greater anti-pseudomonal LPS IgA antibody response than the intermittently or non-colonised patients. However, as with the ELISA studies with sputum, IgA antibodies were found in some of both these latter groups of patients. Statistical analysis of the ELISA results for saliva anti-LPS IgA antibodies showed: i) the difference in OD values between patients chronically colonised with *P. aeruginosa* and non-*P. aeruginosa* colonised patients was highly significant ($P < 0.01$) for PEV-02 S-LPS and very highly significant ($P < 0.001$) for PAC605 R-LPS; ii) the

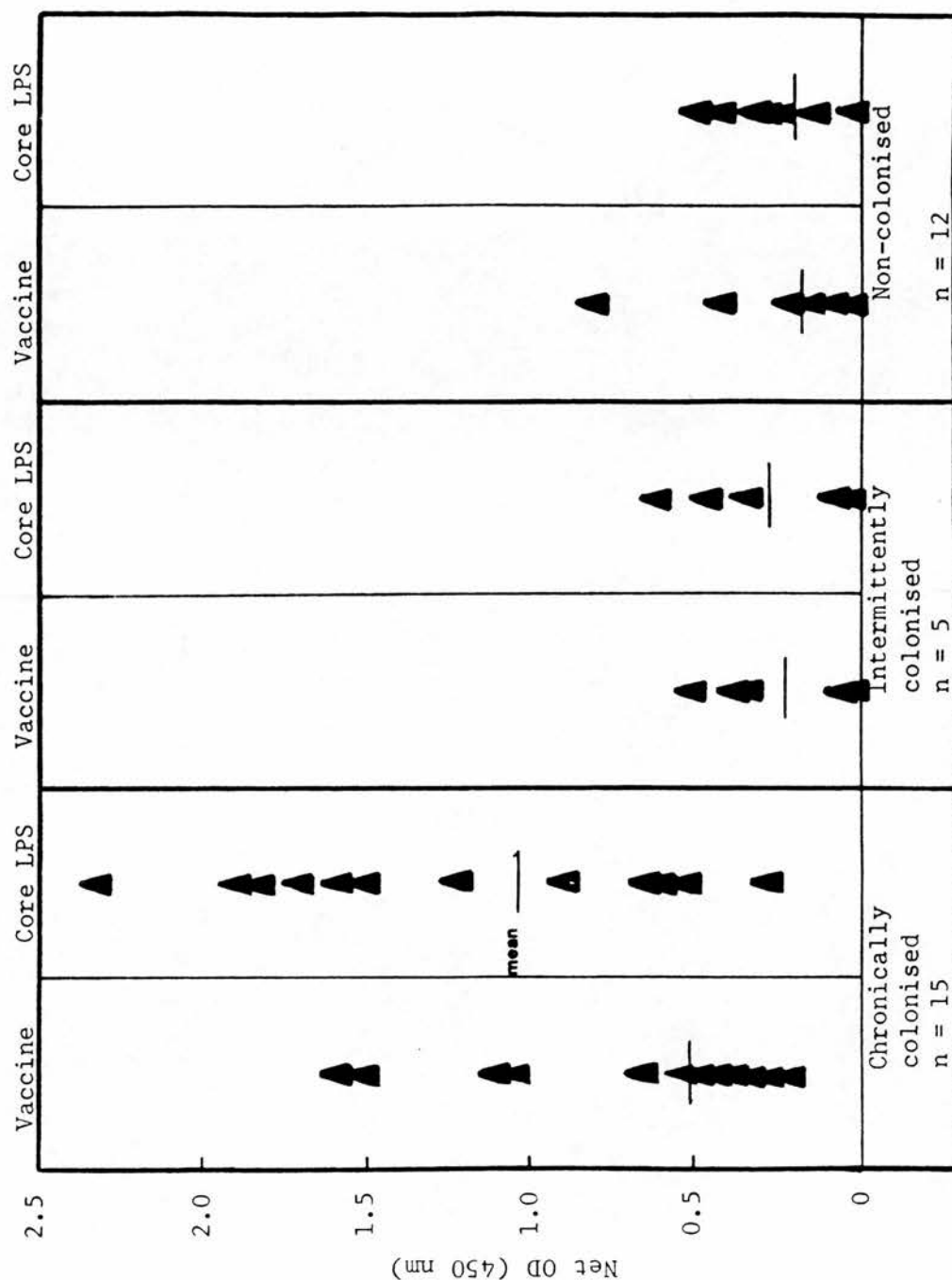


Figure 8. Sputum IgA anti-*P. aeruginosa* LPS antibodies in 32 CF patients measured by ELISA with either heterogeneous polyvalent S-LPS vaccine (PEV-02) or core R-LPS (PAC605) as coating antigens: a total of 15 CF patients chronically colonised with *P. aeruginosa*, 5 intermittently colonised, and 12 non-colonised CF patients are included.

difference between chronically colonised patients and intermittently colonised patients was not significant for antibodies directed to PEV-02 S-LPS, but was highly significant ($P < 0.01$) for IgA antibodies directed to PAC605 R-LPS; iii) there was no significant difference in IgA levels between intermittently and non-*P. aeruginosa* colonised CF patients.

In contrast to sputum, anti-pseudomonal LPS IgG antibodies were detected in saliva samples from patients with CF. A range of levels of anti-pseudomonal IgG antibodies directed to both PEV-02 S-LPS and PAC605 R-LPS were observed in saliva of patients with CF (Figure 10).

The highest levels of IgG anti-LPS antibodies in saliva were found in patients chronically colonised by *P. aeruginosa*. The difference in IgG anti-LPS antibody levels between chronically colonised patients and non-*P. aeruginosa* colonised patients was highly significant ($P < 0.01$) for PEV-02 S-LPS and very highly significant ($P < 0.001$) for PAC605 R-LPS. Differences in the antibody levels between chronically colonised and intermittently colonised patients, and between intermittently and non-colonised patients were not significant by the Student *t* test.

1.5 IMMUNOBLOT ANALYSIS OF SERUM AND SPUTUM AGAINST LPS

The serum IgG and sputum IgA antibody responses to *P. aeruginosa* LPS were further investigated by immunoblotting. LPS from proteinase K digests of *P. aeruginosa* whole cells was separated by SDS-PAGE, and electrophoretically transferred to NIC paper for subsequent probing with serum and sputum from patients with CF. The following *P. aeruginosa* LPS preparations were used in each gel for subsequent immunoblot analysis: i) the CF patients own non-mucoid and/or mucoid *P. aeruginosa* strains which had previously been serotyped by standard typing sera; ii) *P. aeruginosa* standard serotype strains included, where appropriate, the serotype equivalent to the patient's strain; the

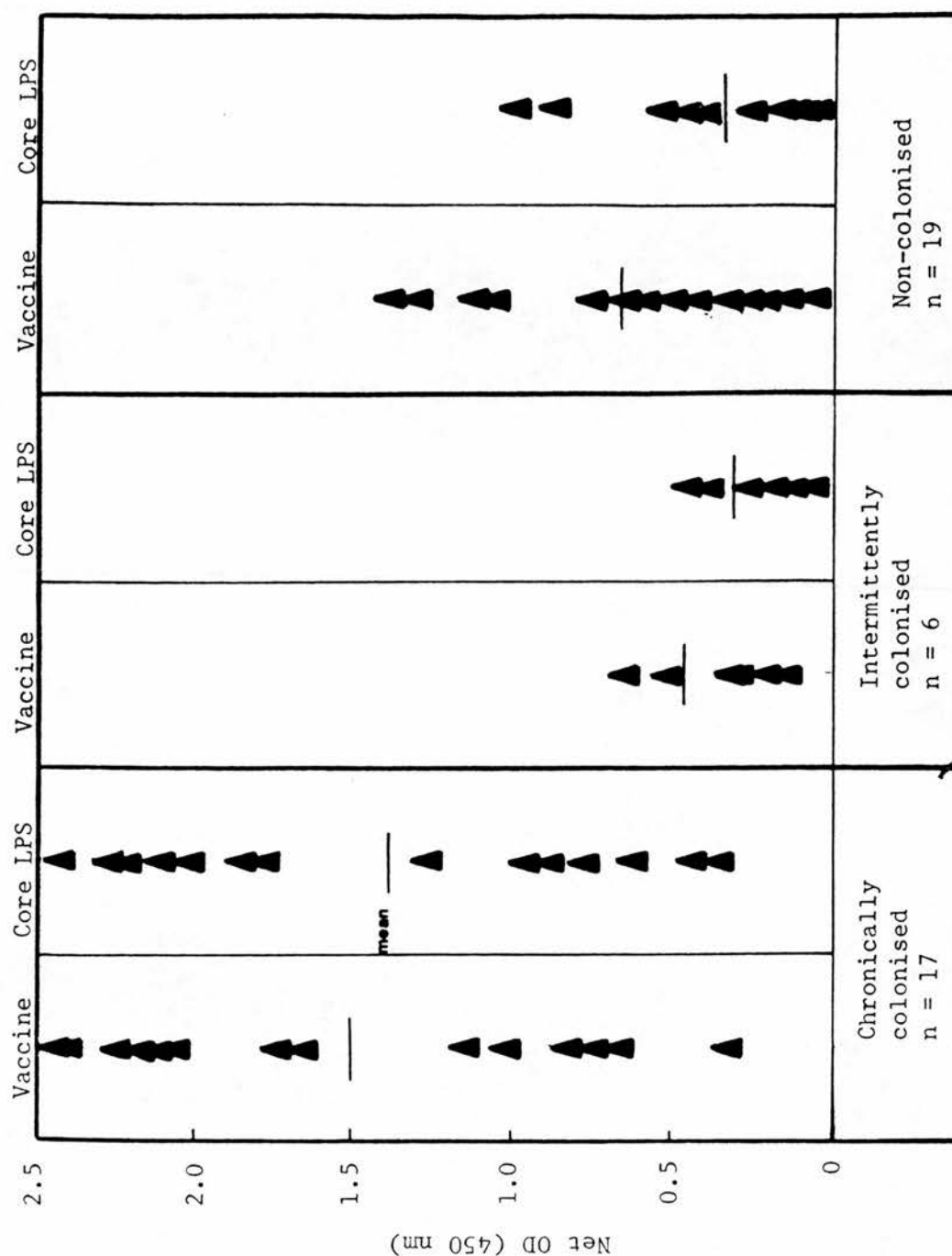


Figure 9. Saliva IgA anti-*P. aeruginosa* LPS antibodies in 42 CF patients measured by ELISA with either heterogeneous polyvalent S-LPS vaccine (PEV-02) or core R-LPS (PAC605) as coating antigens: a total of 17 CF patients chronically colonised with *P. aeruginosa*, 6 intermittently colonised, and 19 non-colonised CF patients are included.

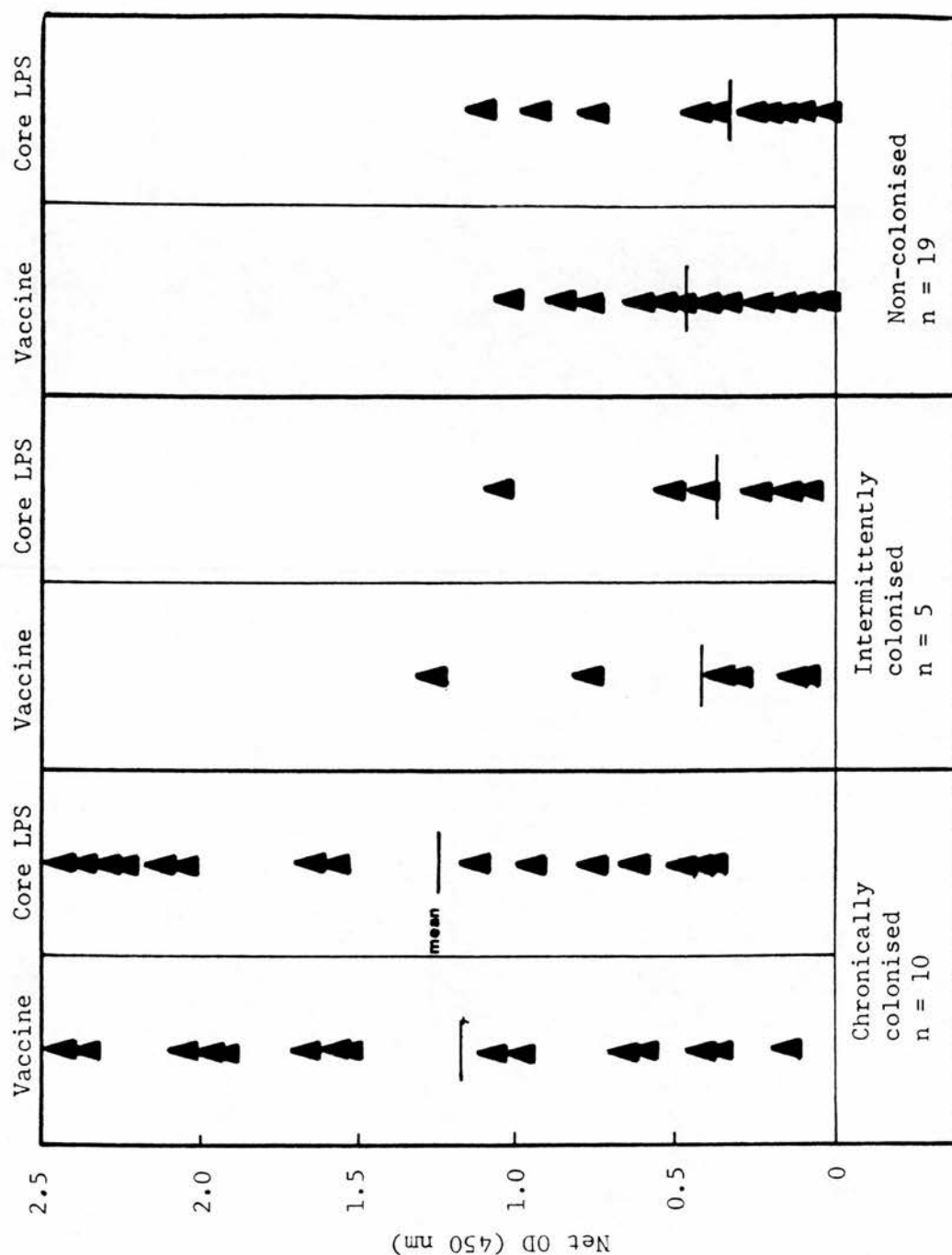


Figure 10. Saliva IgG anti-*P. aeruginosa* LPS antibodies in 39 CF patients measured by ELISA with either heterogeneous polyvalent S-LPS vaccine (PEV-02) or core R-LPS (PAC605) as coating antigens: a total of 15 CF patients chronically colonised with *P. aeruginosa*, 5 intermittently colonised and 19 non-colonised CF patients are included.

serotype strains used included the three most commonly isolated serotypes (O:1, O:6 and O:11) found in Edinburgh as well as some of the less common serotypes; iii) *P. aeruginosa* strains defective in O-antigen production, expressing only core, R-LPS.

The silver stained LPS profiles of six *P. aeruginosa* strains are shown in Figure 11a and include a non-mucoid isolate (serotype O:1) from a CF patient intermittently colonised by the organism. The adjacent photograph (Figure 11b) is the immunoblot probed with serum obtained from the CF patient during the intermittently colonised state. The LPS profile of JN62, the CF patient's strain, exhibits a ladder-like pattern of high molecular weight LPS corresponding to O-antigen substituted LPS. The immunoblot, analysed for anti-*P. aeruginosa* LPS IgG antibodies, shows a positive reaction with bands of high molecular weight, O-antigen LPS of JN62 (Track 1) and the equivalent standard serotype O:1 (Track 6). In addition, a fainter band corresponding to the low molecular weight, common core LPS of these strains was also observed.

Serum taken four months later from the above CF patient, during consistent (isolated on three successive occasions) colonisation by non-mucoid *P. aeruginosa*, was again examined for LPS antibodies by immunoblotting. The LPS profiles of eight *P. aeruginosa* strains are shown in (Figure 12a) together with the subsequent immunoblot (Figure 12b). Serum IgG antibodies directed towards the ladder-like bands of high molecular weight LPS of a number of the S-LPS strains are revealed, including against JN62 - the *P. aeruginosa* isolate from the CF patient (Track 6), and standard serotype strains O:1, O:3 and O:6 (Tracks 1, 2 and 3 respectively). IgG antibodies reactive with the common core LPS of a number of the strains were also observed and included antibodies against core LPS of strains O:1, O:3, O:6, O:8, PAC605, PAC608 and JN62 (Tracks 1, 2, 3, 4, 6, 7 and 8 respectively).

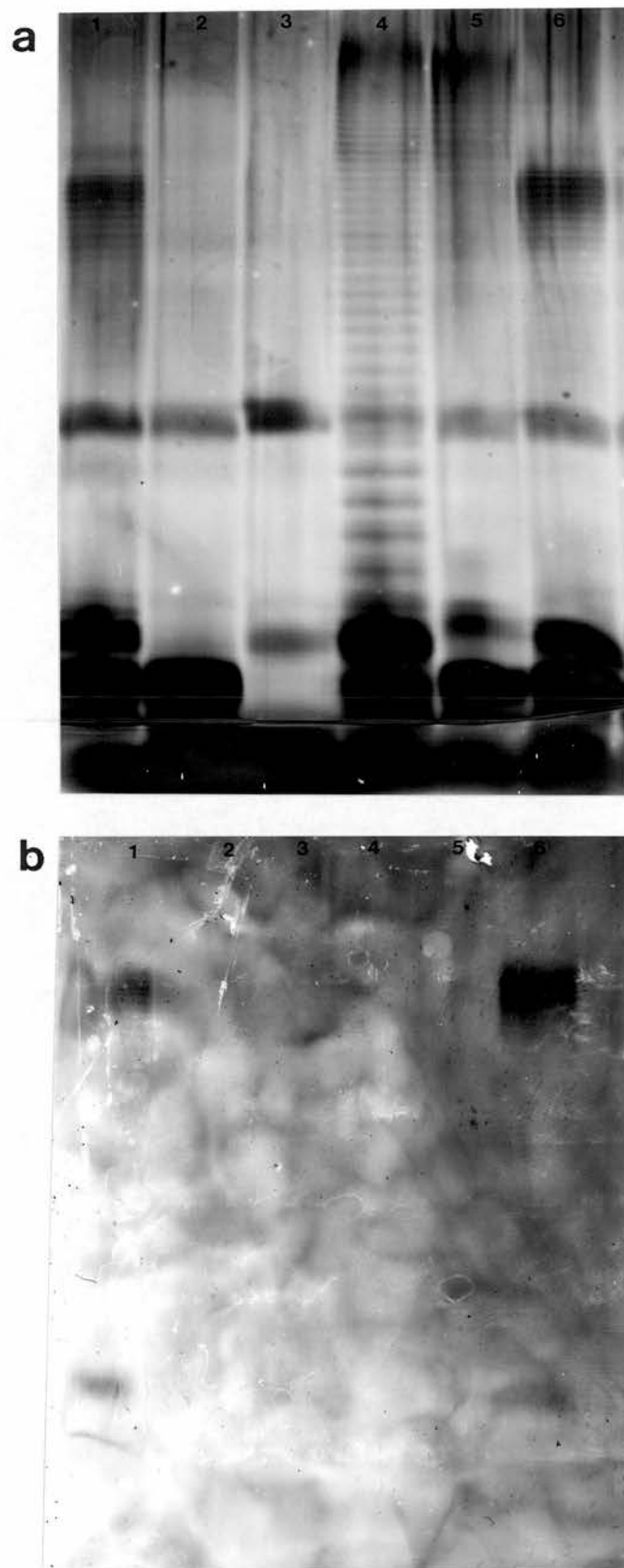


Figure 11. a) Silver stained LPS profiles of proteinase K whole cell digests of 6 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide). b) Immunoblot of anti-*P. aeruginosa* LPS IgG antibodies following transfer of LPS to NIC paper and incubation with serum (1:100) from a CF patient intermittently colonised by non-mucoid *P. aeruginosa*. Track 1, JN62 (CF patient's non-mucoid strain, serotype O:1); Track 2, PAC608 (R-LPS); Track 3, PAC605 (R-LPS); Track 4, serotype O:11 (S-LPS); Track 5, serotype O:6 (S-LPS) and Track 6, O:1 (S-LPS).

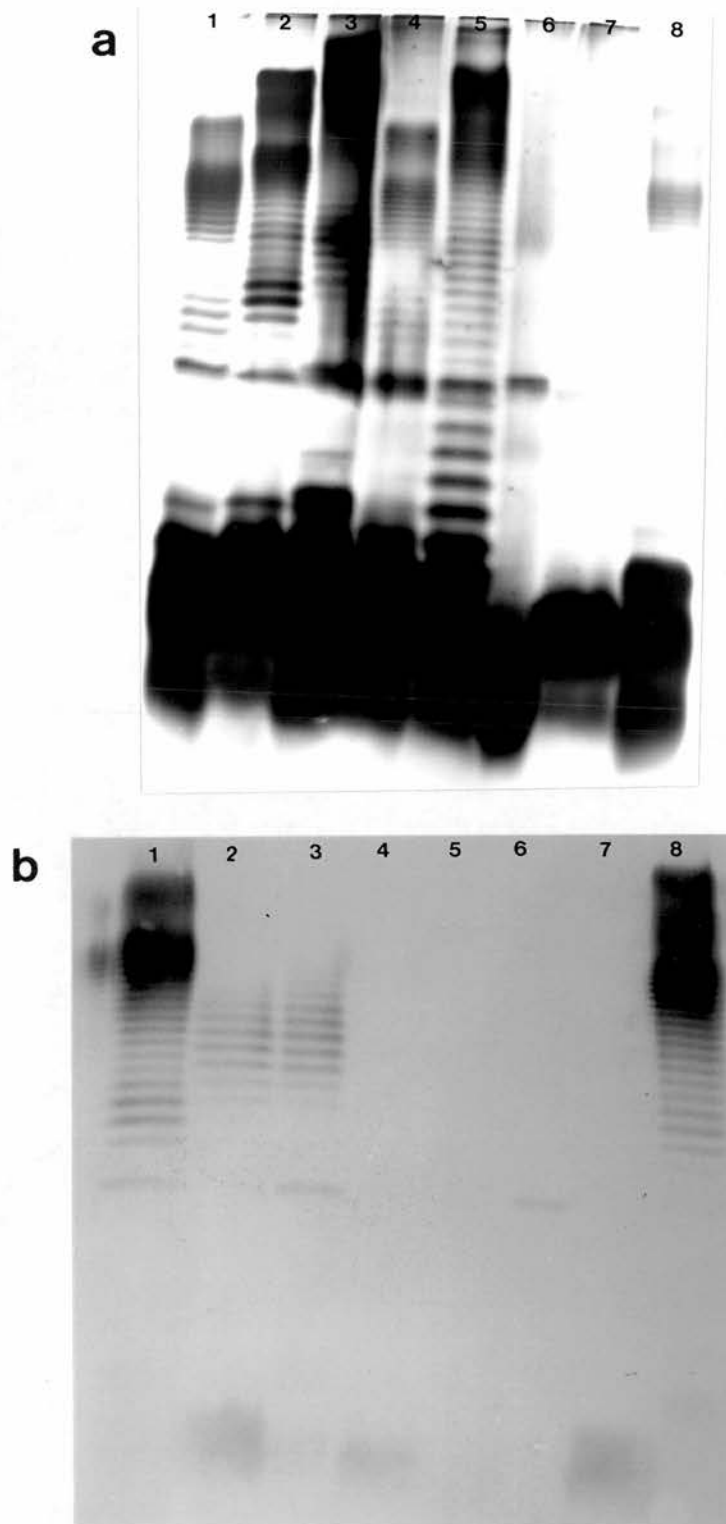


Figure 12. a) Silver stained LPS profiles of proteinase K whole cell digests of 8 *P. aeruginosa* strains b) Immunoblot of anti-*P. aeruginosa* LPS IgG antibodies following transfer of LPS to NIC paper and incubation with serum (1:100) from a CF patient colonised by non-mucoid *P. aeruginosa*. Track 1, serotype O:1 (S- LPS); Track 2, serotype O:3 (S-LPS); Track 3, serotype O:6 (S-LPS); Track 4, serotype O:8 (S-LPS); Track 5, serotype O:11; Track 6, PAC605 (R-LPS); Track 7, PAC608 (R-LPS) and Track 8, JN62 (CF patient's non-mucoid strain).

Serum taken from a young (1 year old) CF patient three months after the first isolation of *P. aeruginosa* from respiratory secretions, was examined for anti-*P. aeruginosa* IgG antibodies by immunoblotting. The LPS profiles of six *P. aeruginosa* strains including the patient's non-mucoid isolate JN61 (serotype O:6) and the immunoblot profiles with the patient's serum are shown in Figure 13a & b. Serum IgG antibodies directed towards the ladder-like bands of high molecular weight O-antigen LPS of the patient's own strain (Track 1) were observed. A reaction with the LPS of the standard serotype O:6 strain was also noted (Track 5) including a faint reactive band corresponding to core LPS.

Sputum and serum from a CF patient colonised with non-mucoid *P. aeruginosa* were analysed by immunoblotting for anti-*P. aeruginosa* LPS IgA and IgG antibodies respectively (Figure 14). Sputum IgA antibodies which reacted with the high molecular weight O-antigen LPS of the patient's strain JN8 (serotype O:6, Track 3) and of serotype O:6 (Track 2) were observed. In addition, a lighter staining region corresponding to IgA antibodies directed towards the faster migrating core LPS of a number of the strains was observed. Serum IgG antibodies reacted with the ladder-like O-antigen LPS of the patient's strain and standard serotype strains O:6 and O:11, Tracks 3, 2 and 5 respectively. A very faint reactive band corresponding to the common core LPS of three strains in Tracks 3, 5 and 7 was also observed.

The serum IgG and sputum IgA antibody responses to *P. aeruginosa* LPS of patients colonised with mucoid *P. aeruginosa* were also examined by immunoblotting. The silver stained LPS profiles of eight *P. aeruginosa* strains are shown in Figure 15 and include a mucoid, non-typable isolate from a CF patient (25 years old). The photographs in Figure 16 show the corresponding immunoblots of the LPS profiles, probed with serum and sputum from the patient. IgG and IgA antibodies, in serum and sputum respectively, directed towards the high molecular weight LPS of some of the strains were found. In addition, strong IgG and IgA antibody responses to the faster migrating

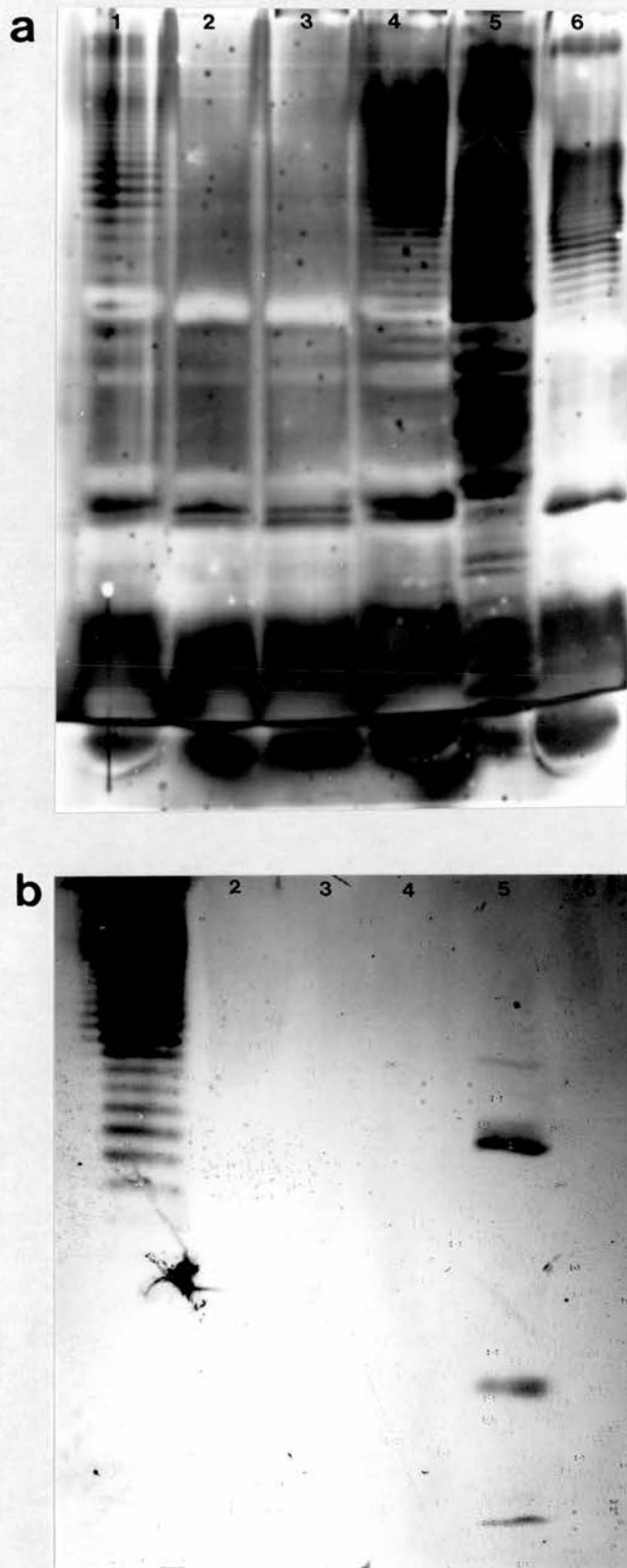


Figure 13. a) Silver stained LPS profiles of proteinase K whole cell digests of 6 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide). b) Immunoblot of anti-*P. aeruginosa* LPS IgG antibodies following transfer of LPS to NIC paper and incubation with serum (1:100) from a CF patient intermittently colonised by non-mucoid *P. aeruginosa*. Track 1, JN61 (CF patient's non-mucoid strain, serotype O:6); Track 2, PAC608 (R-LPS); Track 3, PAC605 (R-LPS); Track 4, serotype O:11 (S-LPS); Track 5, serotype O:6 (S-LPS) and Track 6, serotype O:1 (S-LPS).

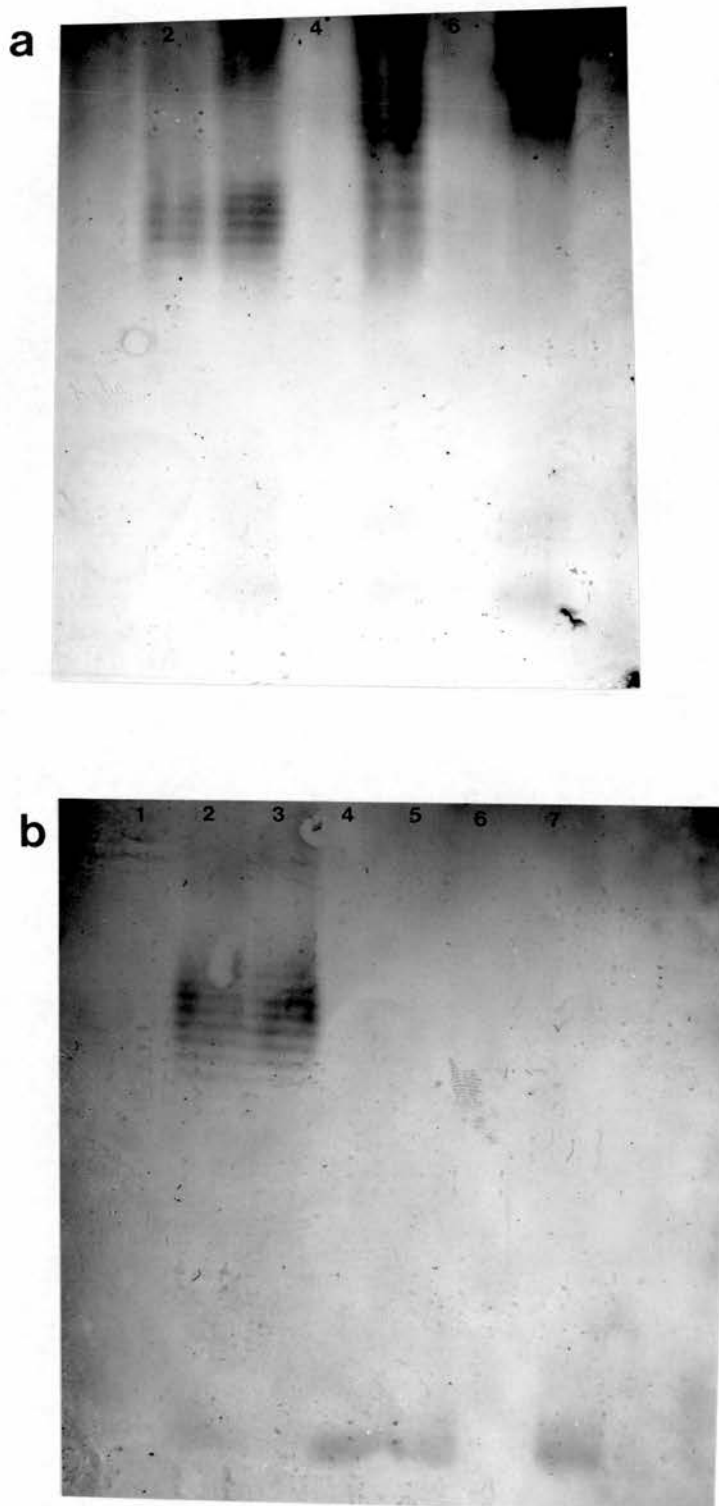


Figure 14. Immunoblots of proteinase K whole cell digests of 7 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with sputum (1:50) and serum (1:200) from a CF patient colonised with non-mucoid *P. aeruginosa*. Blots were analysed for a) sputum IgA and b) serum IgG anti-LPS antibodies. Track 1, serotype O:1 (S-LPS); Track 2, serotype O:6 (S-LPS); Track 3, JN8 (CF patient's non-mucoid strain, serotype O:6); Track 4, serotype O:8 (S-LPS); Track 5, serotype O:11 (S-LPS); Track 6, PAC605 (R-LPS) and Track 7, PAC608 (R-LPS).

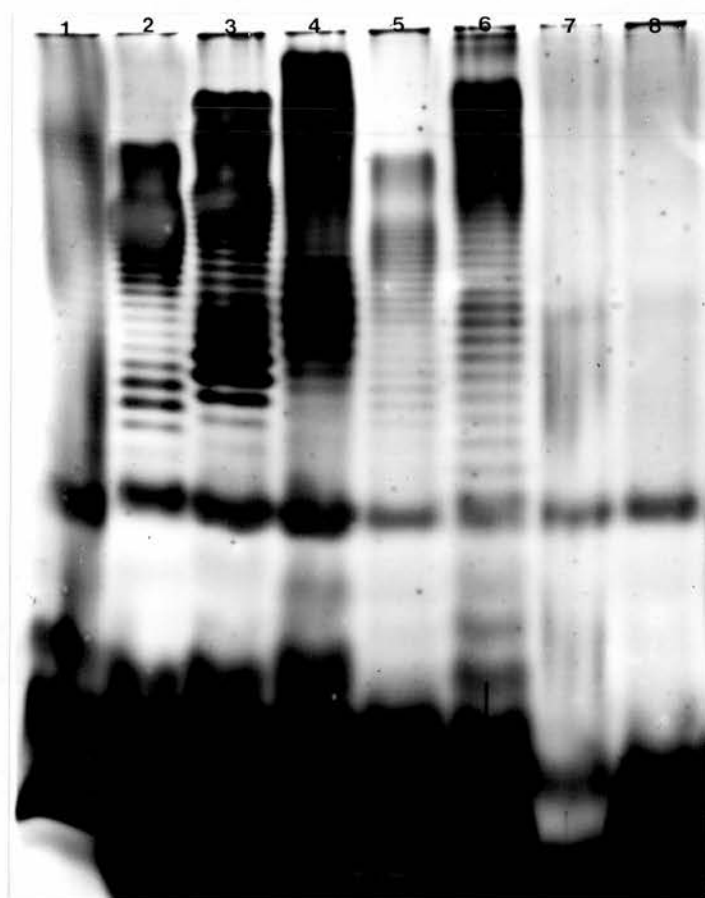


Figure 15. Silver stained LPS profiles of proteinase K whole cell digests of 8 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide). Track 1, JN36 (CF patient's mucoid strain, NT); Track 2, serotype O:1 (S-LPS); Track 3, serotype O:3 (S-LPS); Track 4, serotype O:6 (S-LPS); Track 5, serotype O:8 (S-LPS); Track 6, serotype O:11 (S-LPS); Track 7, PAC605 (R-LPS) and Track 8, PAC608 (R-LPS).

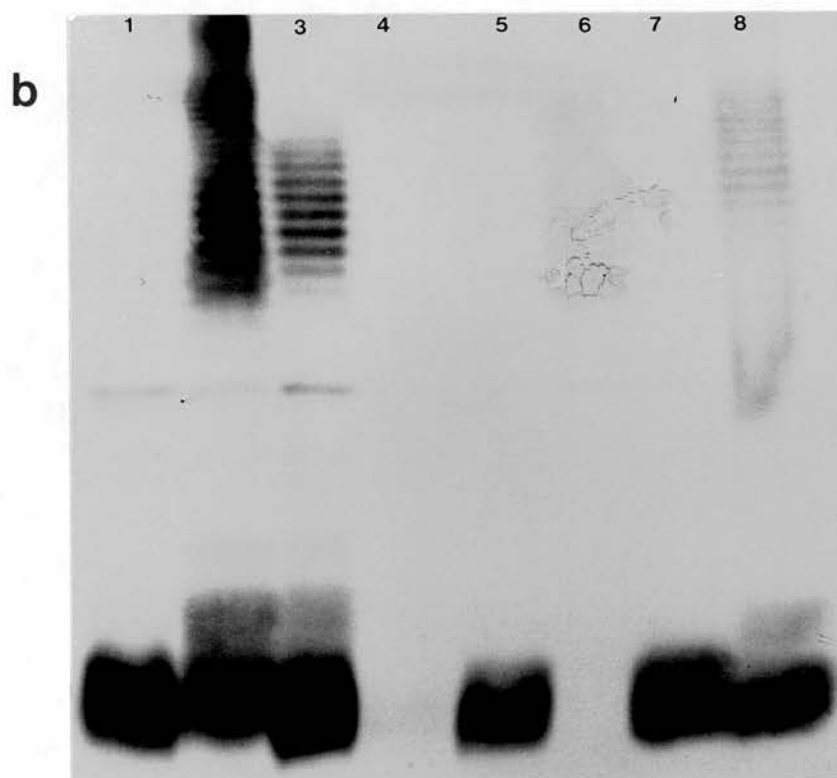
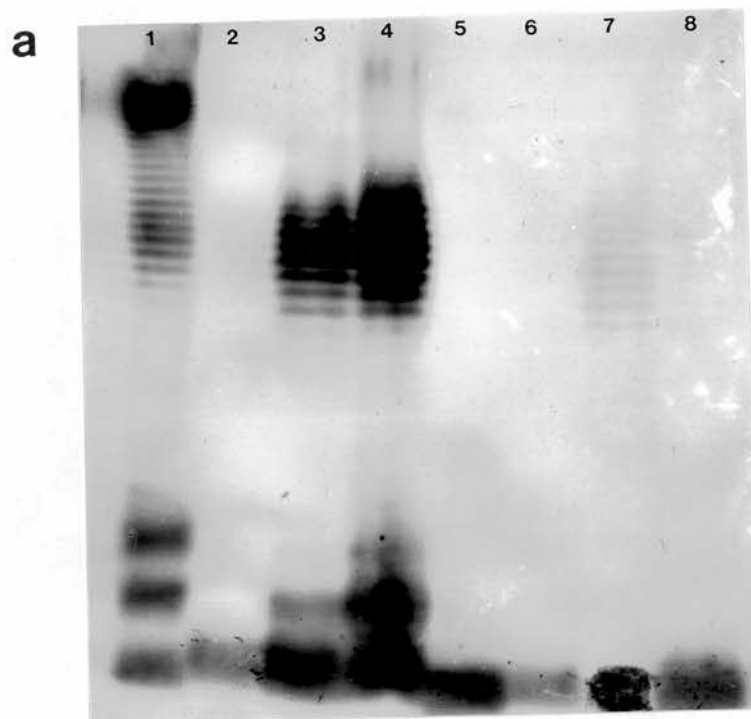


Figure 16. Immunoblots of proteinase K whole cell digests of 8 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with sputum (1:100) and serum (1:200) from a CF patient colonised with mucoid *P. aeruginosa*. Blots were analysed for a) sputum IgA and b) serum IgG anti-LPS antibodies. Track 1, serotype O:1 (S-LPS); Track 2, serotype O:3 (S- LPS); Track 3, serotype O:6 (S-LPS); Track 4, serotype O:8 (S-LPS); Track 5, serotype O:11 (S-LPS); Track 6, PAC605 (R-LPS); Track 7, PAC608 (R-LPS) and Track 8, JN36 (CF patient's mucoid strain NT).

common core LPS of most of the strains were observed. A blot probed with sputum and analysed for IgG antibodies showed an absence of any reactive bands confirming previous results obtained by ELISA (data not shown).

Immunoassay of serum from another CF patient with chronic *P. aeruginosa* infection is shown in Figure 17. The silver stained LPS profiles of proteinase K digests of ten *P. aeruginosa* strains, including the patient's own non-mucoid and mucoid strains (Figure 17a), and the immunoblot analysis with the patient's serum (Figure 17b) are shown. The LPS extracts of the patient's *P. aeruginosa* isolates (Tracks 1 and 2) clearly showed the absence of any of the high molecular weight, ladder-patterned O-antigen LPS characteristic of serotypable, smooth *P. aeruginosa* strains eg serotype O:11 (Track 4), ie the LPS profiles resembled that of PAC605 (Track 3) displaying only the common core R-LPS. In the immunoblot, a band was obtained against the low molecular weight common core LPS of all the *P. aeruginosa* strains analysed. Light bands were also observed with a number of the higher molecular weight O-antigen subunits of some of the S-LPS serotype *P. aeruginosa* strains. In addition, an immunoblot analysed for sputum anti-*P. aeruginosa* LPS IgA antibodies (Figure 18) also showed a reactive band against the common core LPS.

1.6 ABSORPTION OF SERA WITH *P. AERUGINOSA* WHOLE CELLS

The presence of a substantial anti-core LPS antibody component in sera from CF patients chronically colonised by *P. aeruginosa*, shown in the ELISA and immunoblotting studies, was confirmed by absorption experiments (Figure 19). Serum IgG anti-*P. aeruginosa* LPS antibodies in CF patients chronically colonised with *P. aeruginosa* were measured by ELISA before and after a series of absorptions with *P. aeruginosa* whole cells. The reduction in the measurable serum IgG anti- *P. aeruginosa* LPS antibody response after previously absorbing serum with PAC605 (short, incomplete core R-LPS) and PAC608 (complete core R-LPS) indicated the substantial anti-core LPS

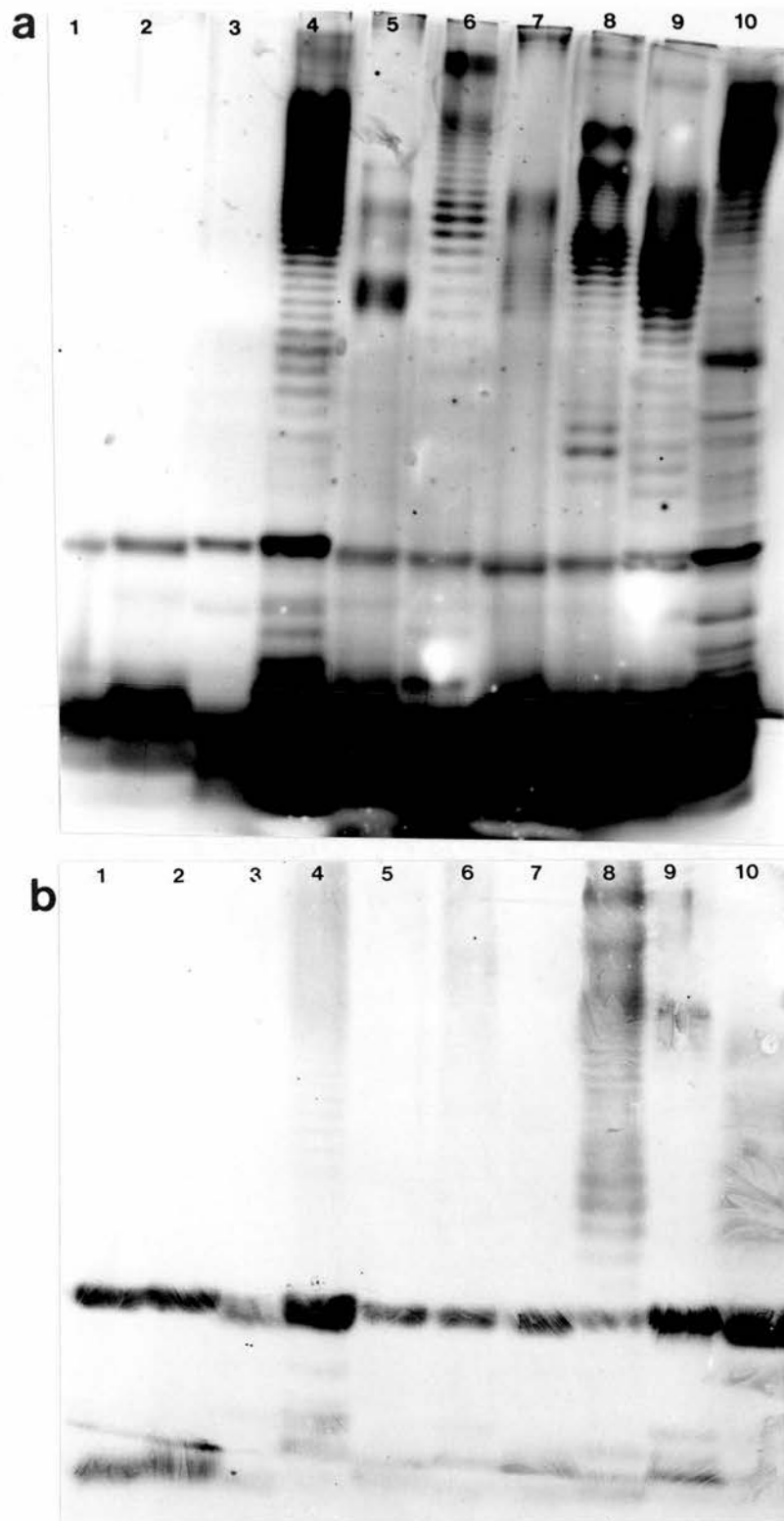


Figure 17. a) Silver stained LPS profiles of proteinase K whole cell digests of 10 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide). b) Immunoblot of anti-*P. aeruginosa* LPS IgG antibodies following transfer of LPS to NIC paper and probed with serum (1:200) from a CF patient chronically colonised with *P. aeruginosa*. Track 1, JN50 (CF patient's mucoid strain, NT); Track 2, JN49 (CF patient's non-mucoid strain, NT); Track 3, PAC605 (R-LPS); Track 4, serotype O:11 (S-LPS); Track 5, serotype O:10 (S-LPS); Track 6, serotype O:9, (S-LPS); Track 7, serotype O:8 (S-LPS); Track 8, serotype O:3 (S-LPS); Track 9, serotype O:1 (S-LPS) and Track 10, serotype O:6 (S-LPS).



Figure 18. Immunoblot of proteinase K whole cell digests of 7 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with sputum from a CF patient chronically colonised with *P. aeruginosa*. The blot was analysed for IgA anti-LPS antibodies. Track 1, JN50 (CF patient's mucoid strain, NT); Track 2, JN49 (CF patient's non-mucoid strain, NT); Track 3, PAC605 (R-LPS); Track 4, serotype O:1 (S-LPS); Track 5, serotype O:4 (S-LPS); Track 6, serotype O:6 (S-LPS) and Track 7, serotype O:11 (S-LPS).

antibody component in sera from CF patients chronically colonised by the organism.

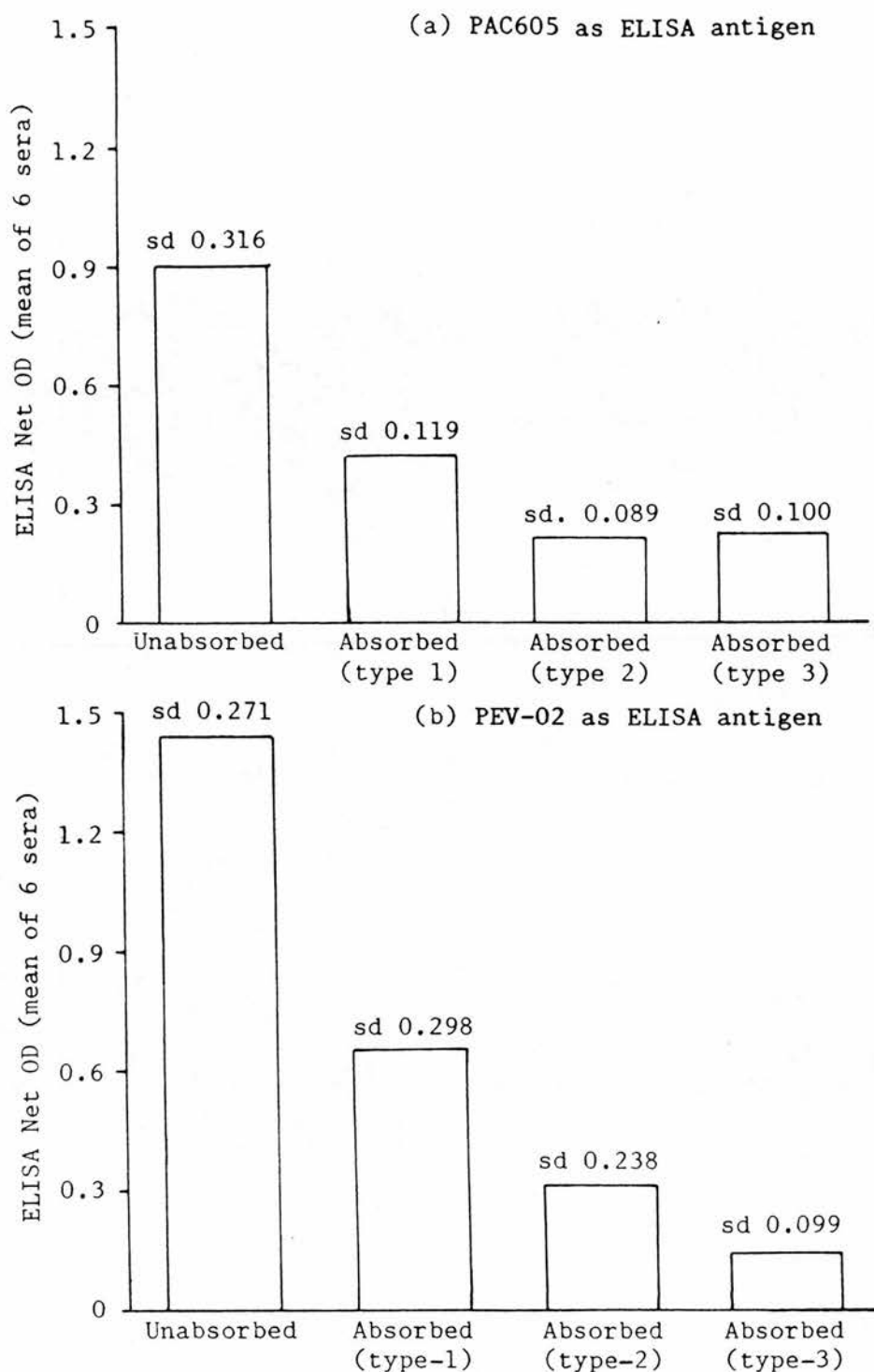


Figure 19. Serum IgG anti-*P. aeruginosa* LPS antibodies in CF patients chronically colonised with *P. aeruginosa* measured by ELISA with (a) R-LPS from PAC605 and (b) S-LPS mixture (PEV-02) as coating antigens before and after a series of absorptions with different strains of *P. aeruginosa* whole washed cells. Unabsorbed sera; type-1, sera absorbed with PAC605; type-2, sera absorbed with PAC605, and then with PAC608; type-3, sera absorbed with PAC605, PAC608, and then a pooled mixture of *P. aeruginosa* serotypes O:1-O:16. OD readings represent the average from 6 individual sera.

CHAPTER 2

DETECTION OF ANTIBODIES TO *P. AERUGINOSA* FLAGELLA

2.1 CHARACTERISATION OF ISOLATED FLAGELLA

Flagellar antigens were prepared from three non-mucoid *P. aeruginosa* strains, J1385, JN61 and JN62 isolated from individual patients with CF. In addition, flagellar antigens were prepared from *P. aeruginosa* PAO1. The flagellar filaments were visualised by electron microscopy after staining with the negative stain phosphotungstic acid (Figure 20) and after shadowing with platinum (Figure 21).

Flagella were characterised by molecular weight determination in SDS-PAGE and by immunoblot analysis with anti-flagellar type-a and -b antisera. Flagella isolated from PAO1, classified as flagella type-b (Allison *et al*, 1985) were used as a standard. Molecular weights of flagella separated by SDS-PAGE (Figure 22) were estimated from a calibration curve of log molecular weight of protein standards against Rf, where Rf is the distance moved by a standard protein through the separating gel divided by the distance moved by bromophenol blue marker. Flagella from strain J1385 (Track 2) appeared to have a similar mobility in SDS-PAGE as flagella from PAO1 (Track 1) and had a calculated M_r of 53,000 characteristic of type-b flagella. The flagella from strains JN61 (Track 3) and JN62 (Track 4) migrated further than PAO1 flagella, with calculated M_r s of 45,000 and 47,000 respectively, and were preliminarily designated as type-a flagella. The presence of a band of low molecular weight protein in the gel profile of flagella from JN61 (Track 3) was noted.

Immunoblot analysis of flagella with anti-flagellar type-a and -b antisera (Figure 23a & b) confirmed the results of SDS-PAGE. The antiserum against type-a flagella reacted strongly with flagella from JN61 (Track 3) and JN62

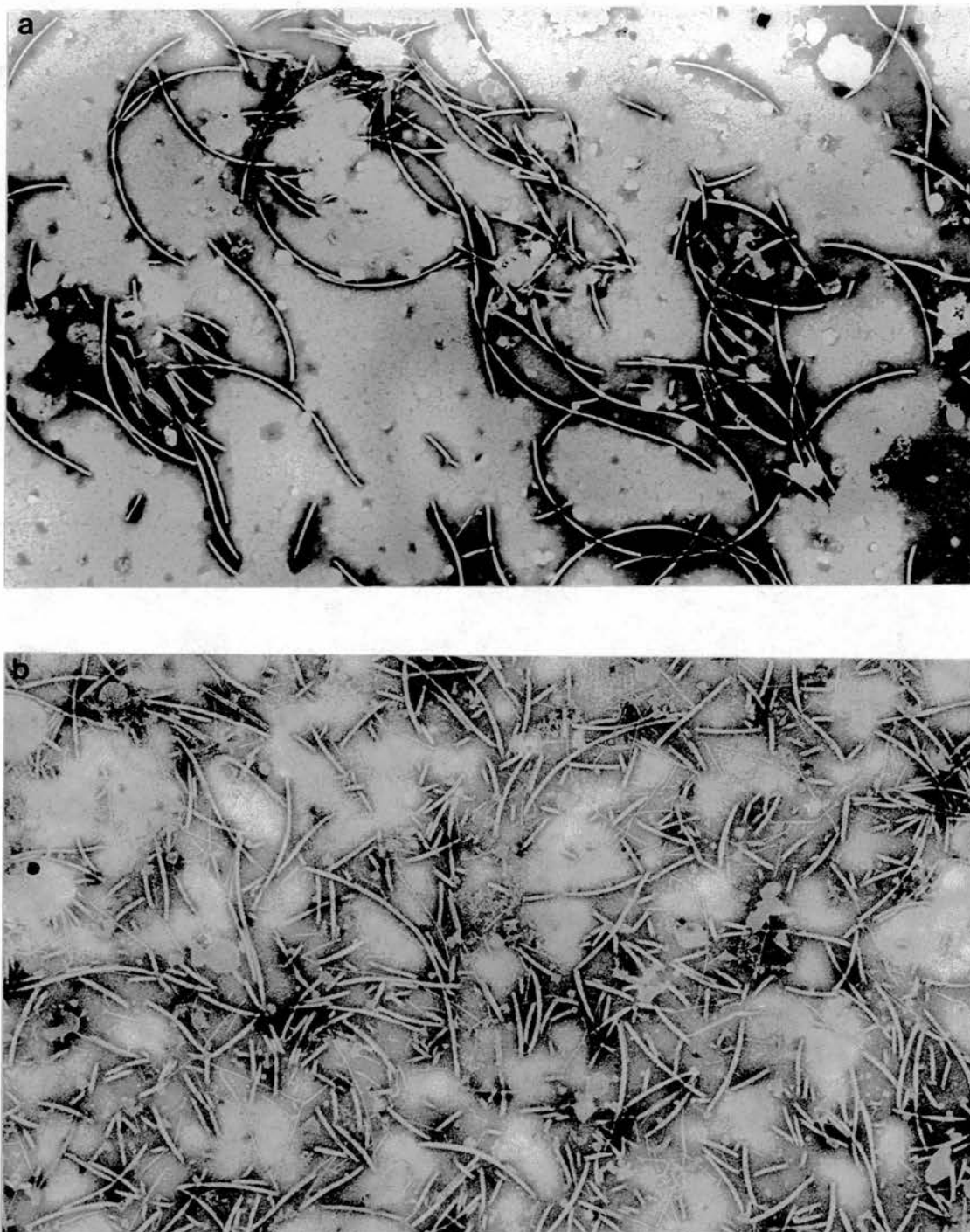


Figure 20. Electron micrographs of flagellar antigen preparations from *P. aeruginosa* strains JN61 (flagellar type-a), (Figure a) and PAO1 (flagellar type-b), (Figure b) stained with 2% w/v phosphotungstic acid. Magnification x 15,000 (Figure a) and x 10,000 (Figure b).

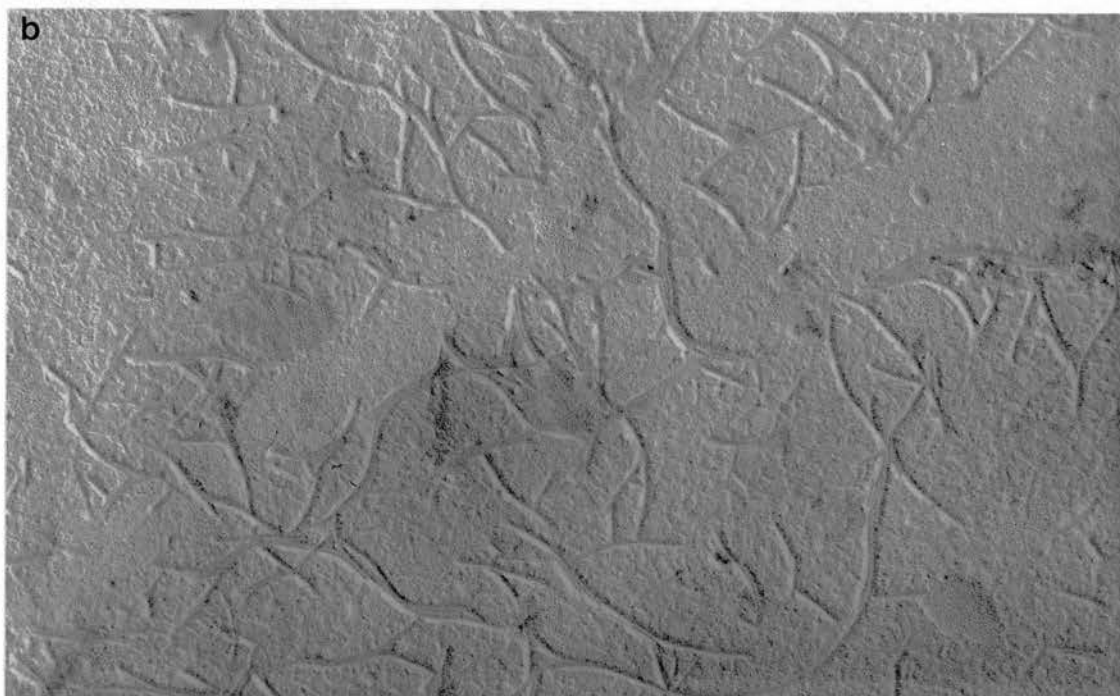
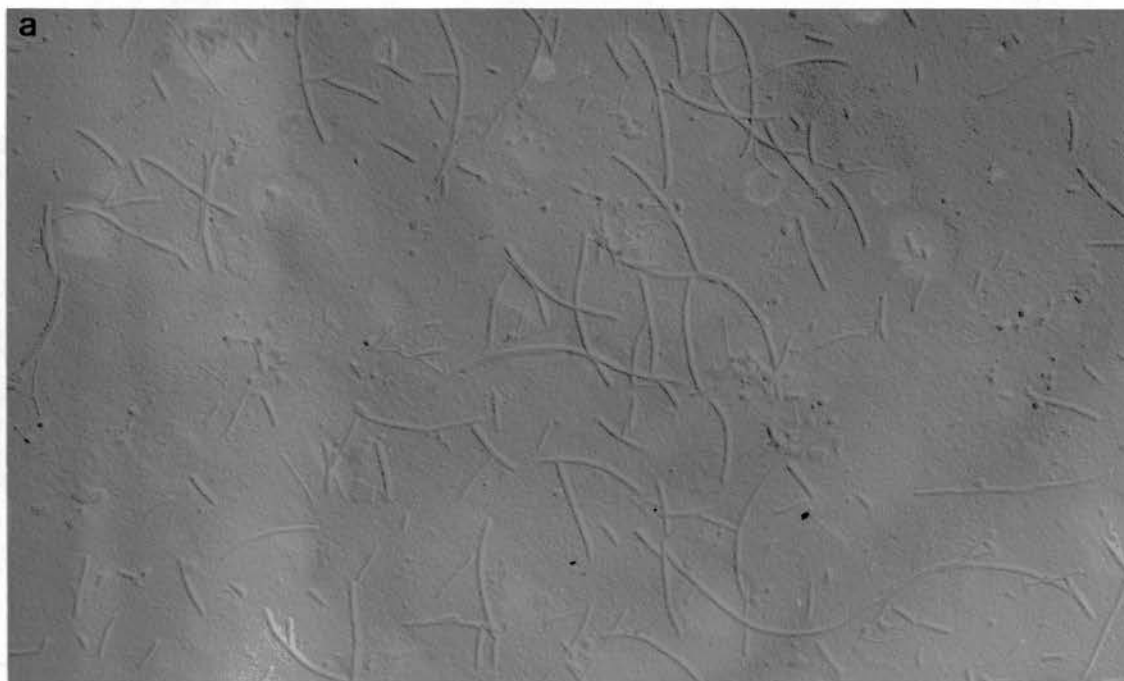


Figure 21. Electron micrographs of flagellar antigen preparations from *P. aeruginosa* strains JN62 (flagellar type-a), (Figure a) and J1385 (flagellar type-b), (Figure b) shadowed with platinum. Magnification x 15,000 (Figure a) and x 12,000 (Figure b).

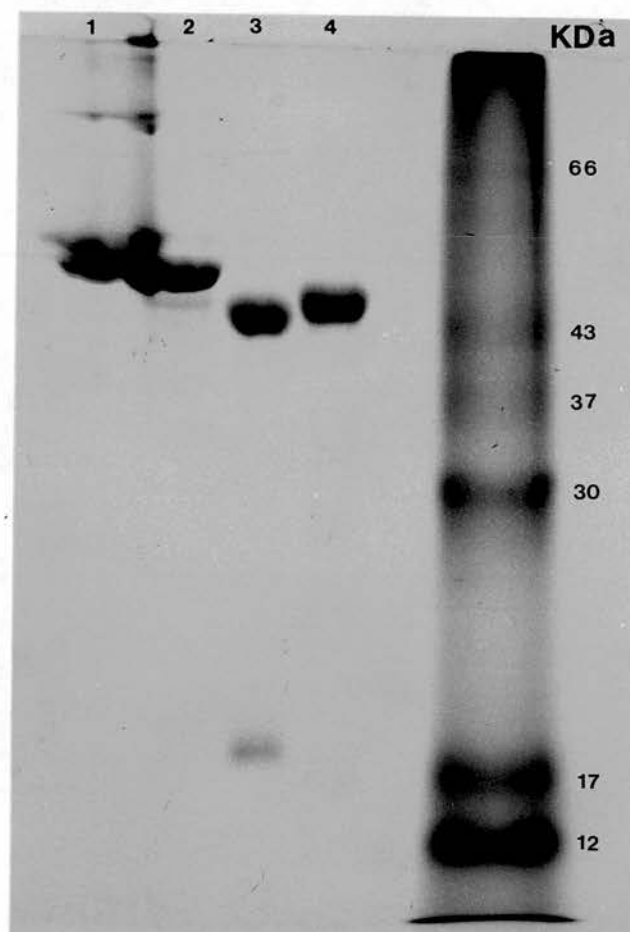


Figure 22. SDS-PAGE of flagella preparations from *P. aeruginosa* strains PAO1 (Track 1), J1385 (Track 2), JN61 (Track 3) and JN62 (Track 4). Flagella were separated using 12% w/v acrylamide gels and visualised with Coomassie blue stain. Molecular weights of protein standards are indicated.

(Track 4) with barely any detectable reaction with flagella from PAO1 (Track 1) and J1385 (Track 2). Flagellar type-b antisera produced strong bands with flagella from PAO1 (Track 1) and J1385 (Track 2) and weaker bands with flagella from JN61 and JN62 (Tracks 3 and 4).

2.2 SERUM ANTI-FLAGELLAR ANTIBODIES DETECTED BY ELISA

Anti-flagellar IgG antibodies were detected by ELISA in sera from patients with CF and in sera from healthy controls (Figure 24). Elevated levels of anti-flagellar IgG antibodies above the control range were found in CF patients intermittently or chronically colonised with *P. aeruginosa*. However, a number of CF patients classified as non-*P. aeruginosa* colonised on the basis of sputum bacteriology also showed increased levels of serum anti-flagellar IgG antibodies. For many of the CF patients a similar IgG antibody response to both type-a and -b flagella was observed. A pronounced antibody response to either type-a or -b flagella was also observed for some CF patients.

Statistical analysis of the results by the Student *t* test revealed: i) the difference in absorbance readings between chronically colonised patients and both non-*P. aeruginosa* colonised CF patients and healthy controls was very highly significant ($P < 0.001$) for type-a and -b flagella; ii) the difference in OD readings between intermittently colonised CF patients and healthy controls was very highly significant ($P < 0.001$) for type-a flagella and highly significant ($P < 0.01$) for type-b flagella; iii) the difference between intermittently and non-*P. aeruginosa* colonised patients was highly significant ($P < 0.01$) for type-a flagella and significant for type-b flagella; iv) differences between intermittently and chronically colonised patients, and between non-colonised CF patients and controls were not significant.

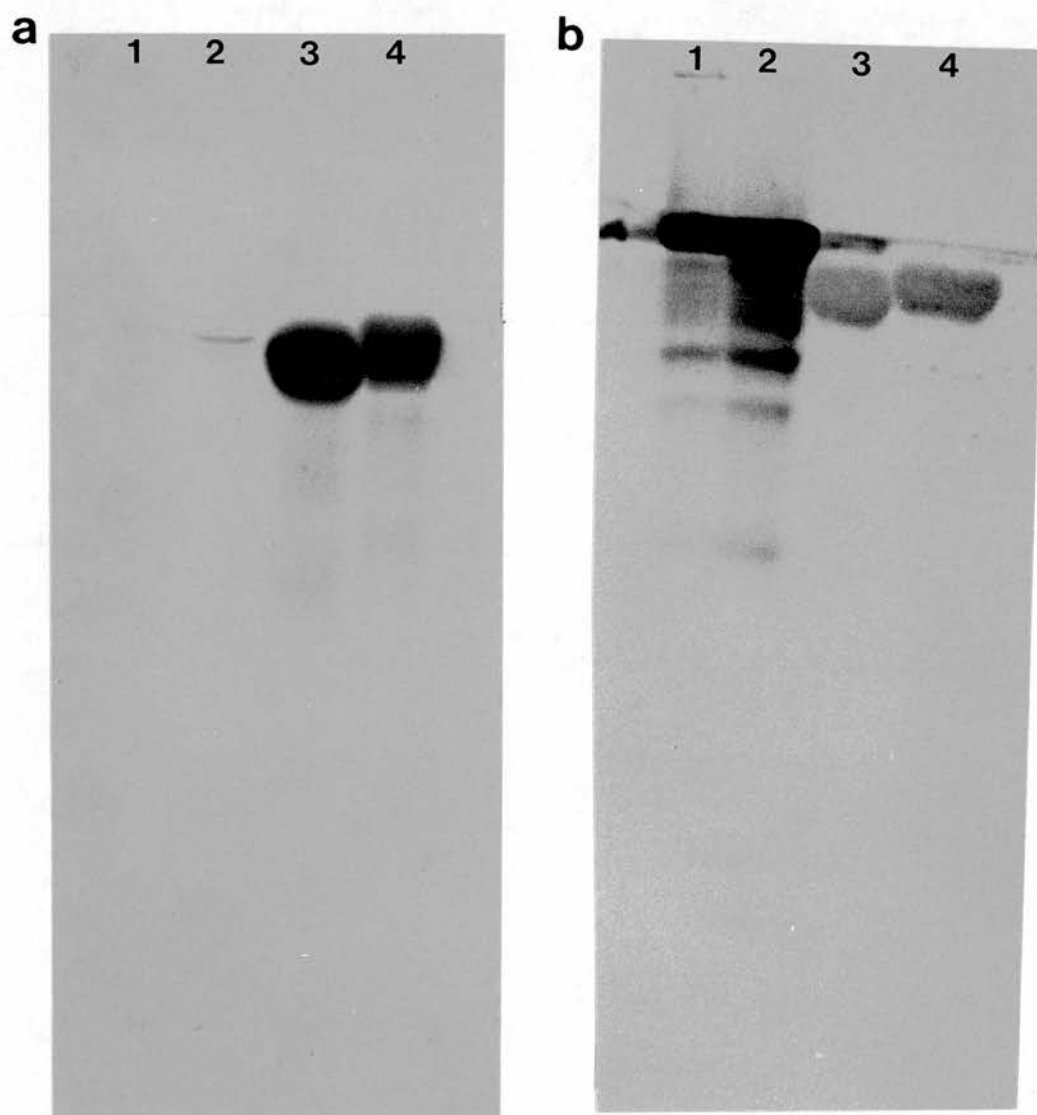


Figure 23. Immunoblots of flagellar antigens separated by SDS-PAGE followed by electrophoretic transfer to NIC paper and probed with anti-flagellar type-a antisera (a) and anti-flagellar type-b antisera (b): flagellar antigens from PAO1 (Track 1); J1385 (Track 2), JN61 (Track 3) and JN62 (Track 4).

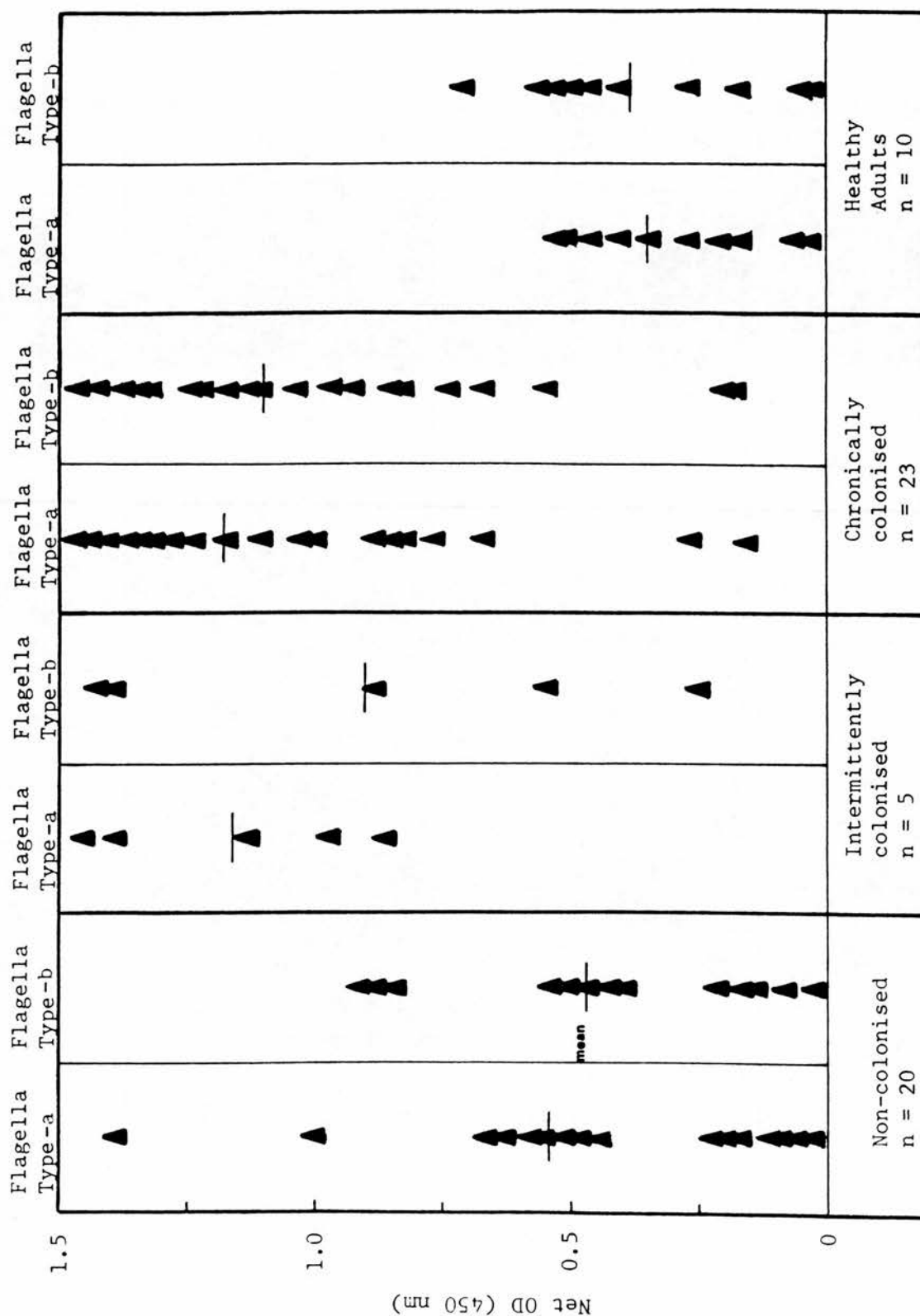
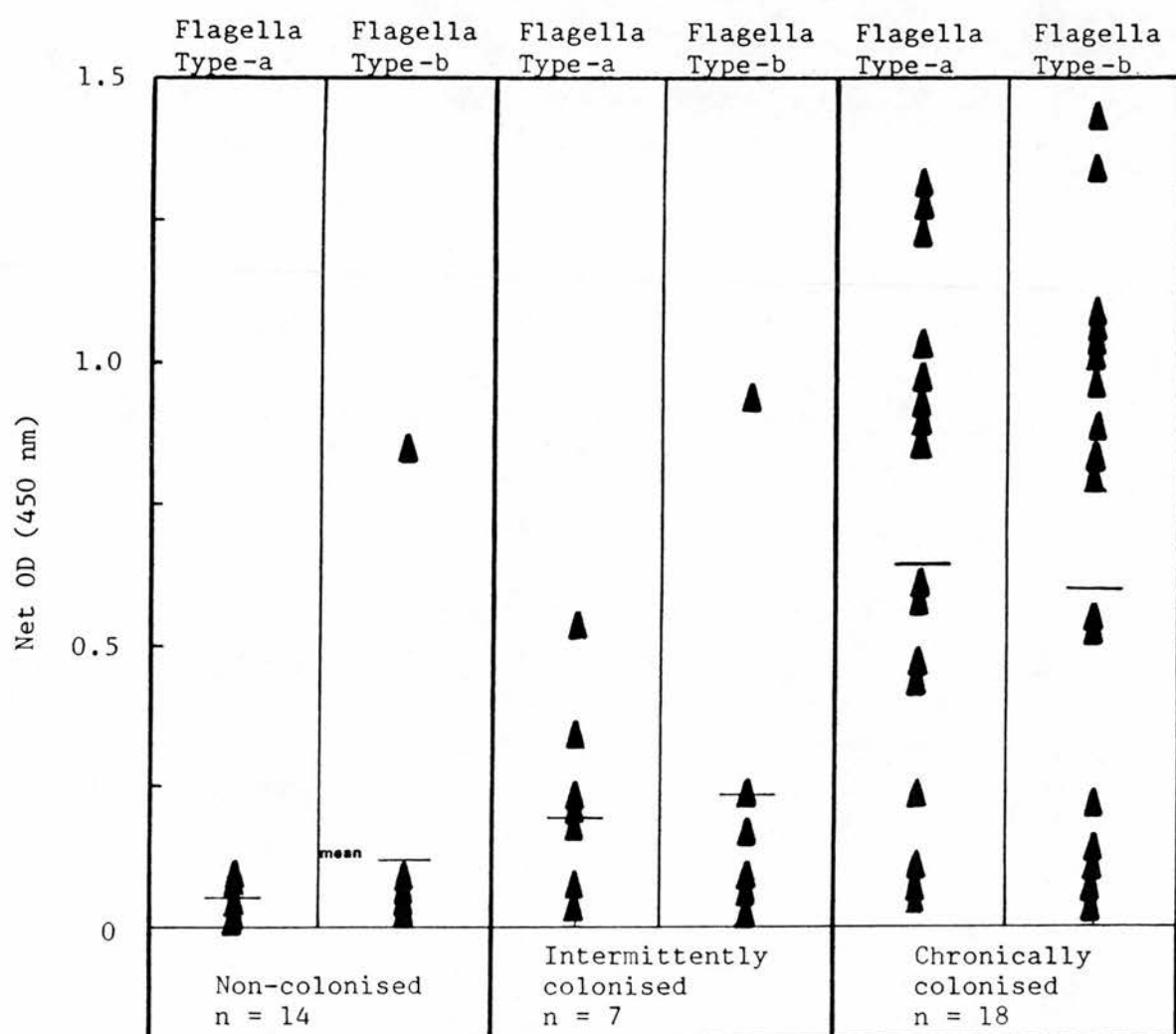


Figure 24. Serum IgG anti-flagellar antibodies 48 CF patients and 10 non-CF individuals measured by ELISA. A total of 23 CF patients chronically colonised with *P. aeruginosa*, 5 intermittently colonised, 20 non-colonised CF patients and 10 healthy adults are included.

2.3 SPUTUM AND SALIVA ANTI-FLAGELLAR ANTIBODIES DETECTED BY ELISA

Anti-flagellar IgA antibodies were also detected by ELISA in sputum (Figure 25) and saliva (Figure 26) from a number of patients with CF who were chronically or intermittently colonised with *P. aeruginosa*. A range of anti-flagellar IgA antibodies in these two groups of patients were detected; a net absorbance value >0.1 was classed as positive for anti-flagellar antibody. In some patients a similar IgA response to both flagellar types was noted whilst in other patients a preponderance of IgA antibodies to either type-a or -b flagella was observed. Significant anti-flagellar IgA antibodies were not observed in saliva or sputum from CF patients with no previous history of *P. aeruginosa* colonisation. However, there was one exception in the case of an individual who demonstrated sputum IgA antibodies directed towards type-b flagella (OD = 0.840).

Statistical analysis (Student *t* test) of the ELISA results for sputum and saliva anti-flagella IgA antibodies showed: i) the difference between chronically colonised patients and non-*P. aeruginosa* colonised patients for sputum anti-flagellar IgA antibodies was very highly significant ($P < 0.001$) for type-a and -b flagellar antigens; in the case of saliva the difference was significant ($P < 0.05$); ii) the difference between intermittently colonised and non-*P. aeruginosa* colonised patients for sputum anti-flagellar type-a and -b IgA antibodies was highly significant ($P < 0.01$); the difference, for the saliva results was not significant; iii) the difference between intermittently colonised and chronically colonised patients for sputum and saliva anti-flagella IgA antibodies was not significant.



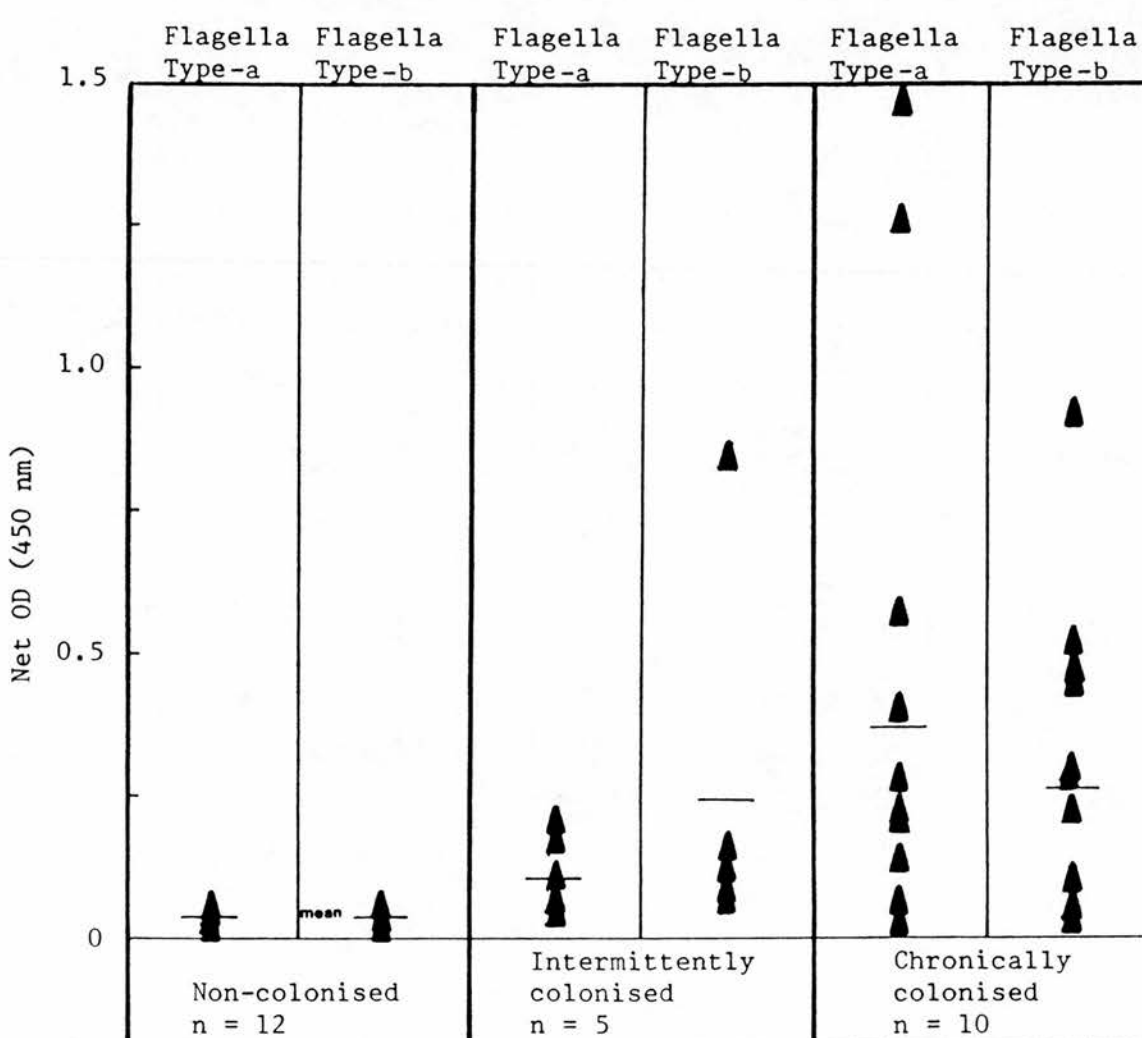


Figure 26. Saliva IgA anti-*P. aeruginosa* flagellar antibodies in 27 CF patients measured by ELISA. A total of 10 CF patients chronically colonised with *P. aeruginosa*, 5 intermittently colonised and 12 non-colonised CF patients are included.

2.4 IMMUNOBLOT ANALYSIS OF SERA, SALIVA AND SPUTUM AGAINST FLAGELLAR ANTIGENS

Immunoblot analysis of sera, saliva and sputa from patients with CF against *P. aeruginosa* flagellar antigen types-a and -b was performed (Figures 27 & 28). The immunoblot studies reflected the results obtained in ELISA. Positive blots, with a response to either one or both *P. aeruginosa* flagellar types, were obtained with sera, saliva or sputa which had conspicuously elevated anti-flagellar IgG/IgA antibodies in ELISA studies. Immunoblot profiles positive for serum IgG anti-flagellar type-a and/or -b antibodies are shown in Figure 27. Positive blots from:- i) healthy control (Strip 1); ii) two non-*P. aeruginosa* colonised patients (Strips 2 and 3); iii) five CF patients intermittently or consistently colonised with non-mucoid *P. aeruginosa* (Strips 4-8) and iv) four CF patients chronically colonised with *P. aeruginosa* (Strips 9-12) are displayed.

Similarly immunoblot profiles positive for saliva or sputum IgA anti-flagellar antibodies are shown in Figure 28. A weak, detectable anti-flagellar (type-b) IgA antibody response in a non-*P. aeruginosa* colonised patient is shown in Strip 1; the antibody response to flagellar antigens, in saliva or sputum from patients intermittently or consistently colonised by non-mucoid *P. aeruginosa* (Strips 2-5) and patients chronically colonised by mucoid *P. aeruginosa* (Strips 6-8) are also displayed.

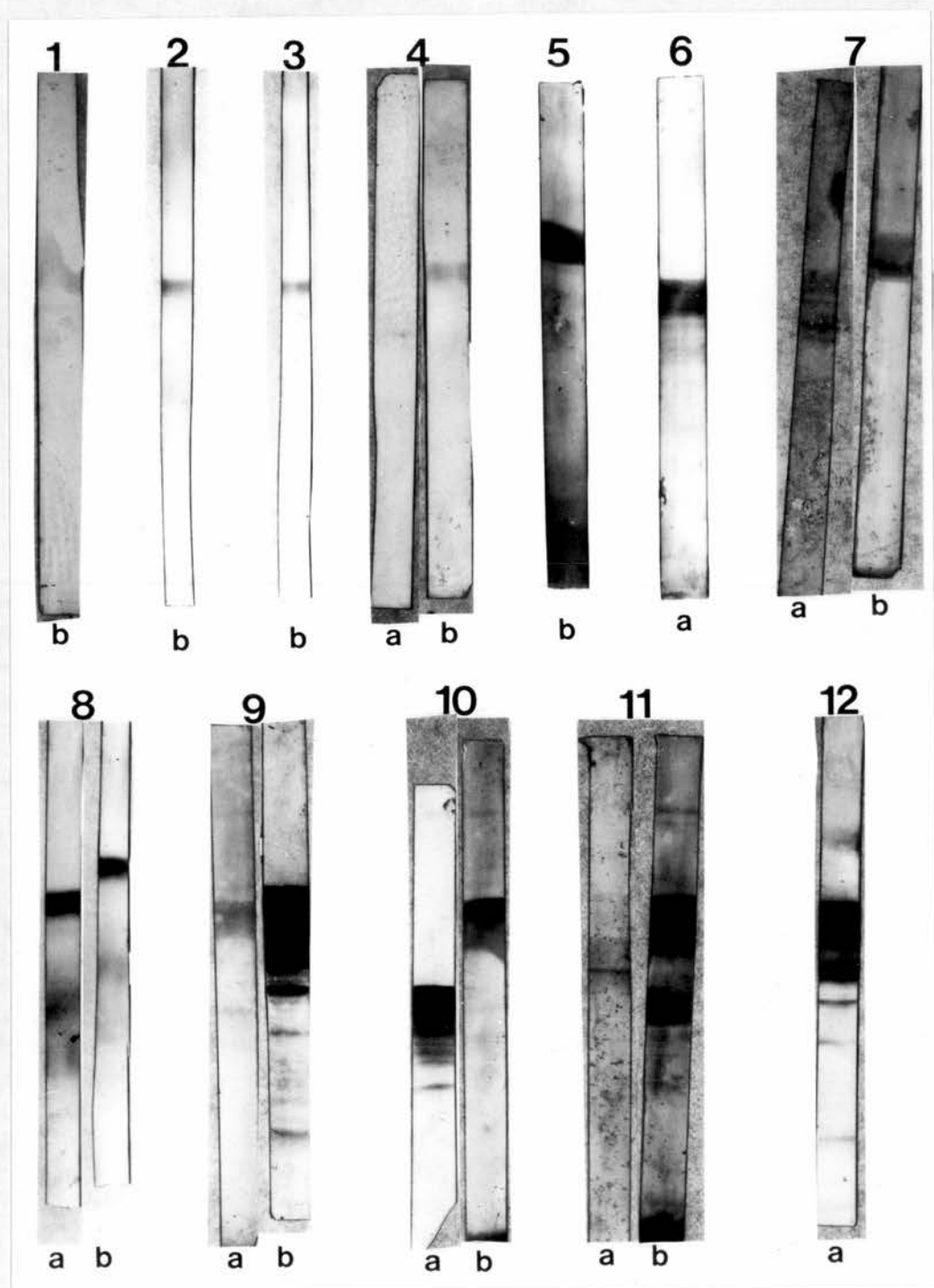


Figure 27. Strip immunoblots of isolated *P. aeruginosa* flagella (type-a and/or type-b) reacted with serum from patients with CF and analysed for the presence of anti-flagellar IgG antibodies. Strip 1, healthy control; Strip 2, CF non-col.; Strip 3, CF non-col.; Strip 4, CF col.; Strip 5, CF col.; Strip 6, CF int. col.; Strip 7, CF int. col.; Strip 8, CF int. col.; Strip 9, chron. col.; Strip 10, chron. col.; Strip 11, chron. col.; Strip 12, chron. col.
 (CF non-col. = CF patient non-*P. aeruginosa* colonised;
 CF col. = CF patient colonised with non-mucoid *P. aeruginosa*;
 CF int. col. = CF patient intermittently colonised with non-mucoid *P. aeruginosa*;
 CF chron. col. = CF patient chronically colonised with mucoid *P. aeruginosa*).

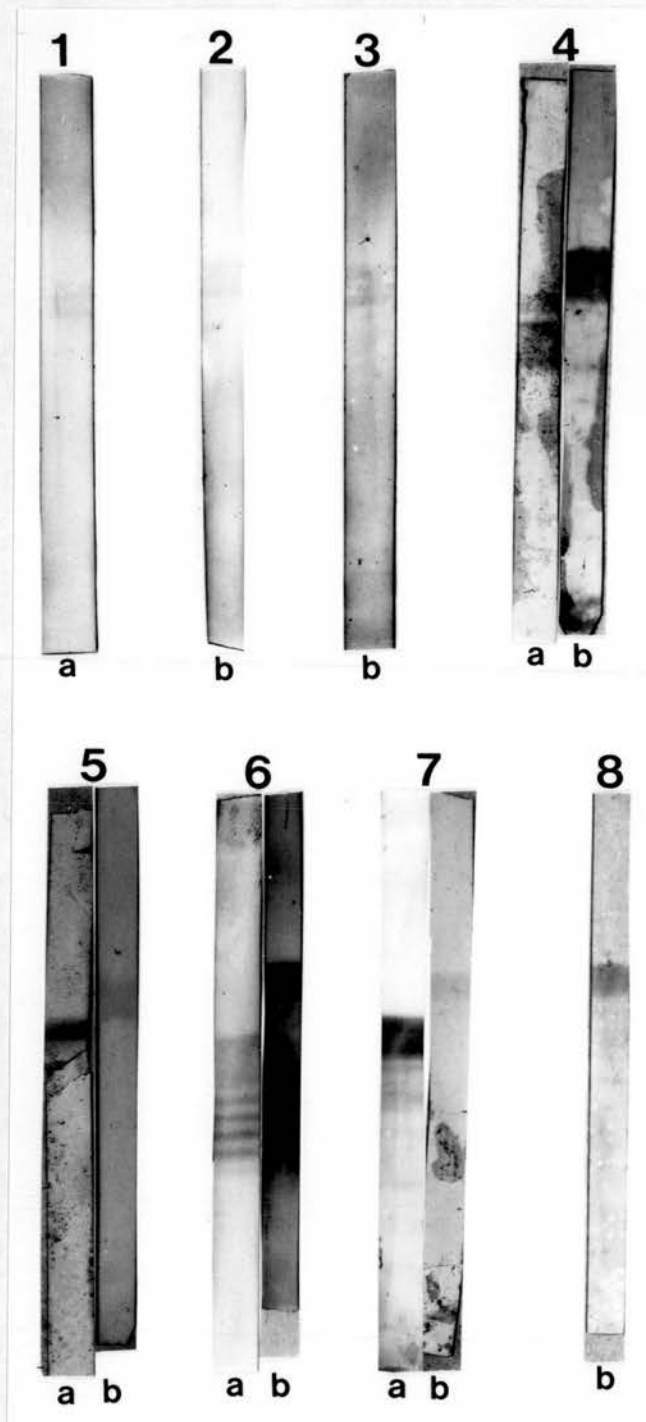


Figure 28. Strip immunoblots of isolated *P. aeruginosa* flagella (type-a and/or type-b) reacted with saliva or sputum from patients with CF and analysed for the presence of anti-flagella IgA antibodies. Strip 1, CF non-col. (Sputum); Strip 2, CF int. col. (Sputum); Strip 3, CF int. col. (Saliva); Strip 4, CF int. col. (Sputum); Strip 5, CF int. col. (Sputum); Strip 6, chron. col. (Sputum); Strip 7, chron. col. (Sputum); Strip 8, chron. col. (Saliva).
 (CF non-col. = CF patient non-*P. aeruginosa* colonised;
 CF int. col. = CF patient intermittently colonised with non-mucoid *P. aeruginosa*;
 CF chron. col. = CF patient chronically colonised with mucoid *P. aeruginosa*).

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF ANTI-*P. AERUGINOSA* LPS MONOCLONAL ANTIBODIES

The initial aim of this work was to produce Mabs against *P. aeruginosa* LPS antigen, with the antibodies able to recognise all O-antigenic serotypes of *P. aeruginosa*. The ultimate aim was to use the Mabs in a capture ELISA system for the detection of *P. aeruginosa* LPS in CF respiratory secretions (eg saliva or sputum) as a means of facilitating early identification of respiratory colonisation by non-mucoid *P. aeruginosa*.

3.1 VACCINATION OF MICE AND SCREENING OF TAIL BLEEDS

Five female **BALB/c** mice were inoculated with heat-killed whole cell preparations of *P. aeruginosa* strains PAC608 and PAC605. These LPS defective mutants, unable to produce serologically distinct O-antigenic side chains, express only core LPS (Figure 29) common to all *P. aeruginosa* serotypes. Mice were vaccinated (as described in Materials and Methods), and blood samples taken for subsequent screening of serum anti-LPS antibodies in ELISA. The serum anti-*P. aeruginosa* LPS antibody response in one mouse (codename Angel), 38 days after initial vaccination, is shown in Figure 30. Serum was screened against: i) core R-LPS extracted from six LPS defective *P. aeruginosa* mutants (see Figure 29 for LPS profiles). PAC605 represents the shortest core expressed by the mutants whilst PAC557 represents the most complete core, the other four PAC mutants having core structures of intermediate size (Rowe & Meadow, 1983); ii) O-antigenic S-LPS from three serotype strains of *P. aeruginosa* (O:1, O:2 and O:11). A positive antibody response (ELISA OD>0.1) against all the *P. aeruginosa* LPS antigens used in the screen was noted. Particularly strong antibody responses to the core R-LPS of PAC611, PAC556 and PAC609 were observed. Elevated antibody levels

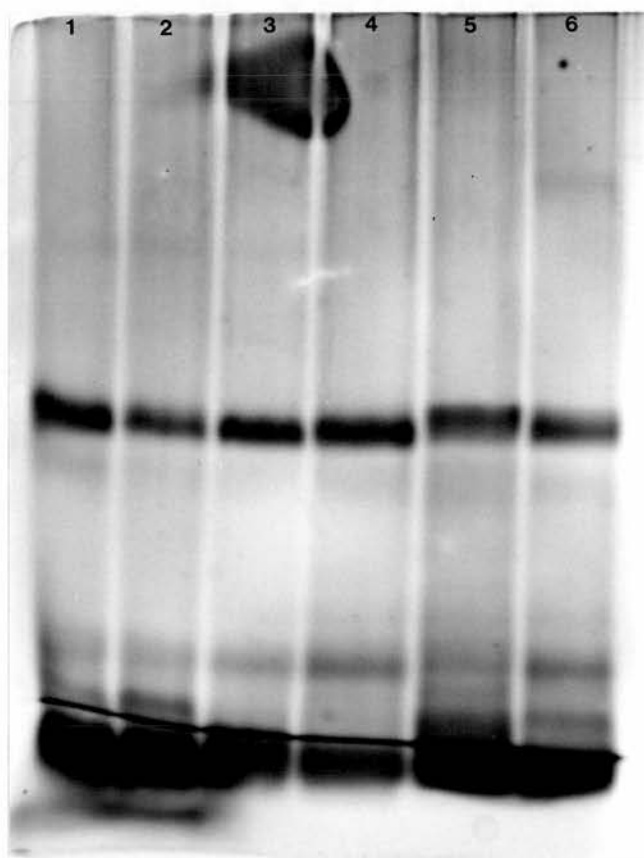


Figure 29. Silver-stained SDS-polyacrylamide gel (14%) of LPS extracted from 6 LPS defective mutants of *P. aeruginosa*. The profiles of PAC557, PAC608, PAC605, PAC611, PAC556 and PAC609 are shown in Tracks 1-6 respectively.

ELISA LPS ANTIGENS

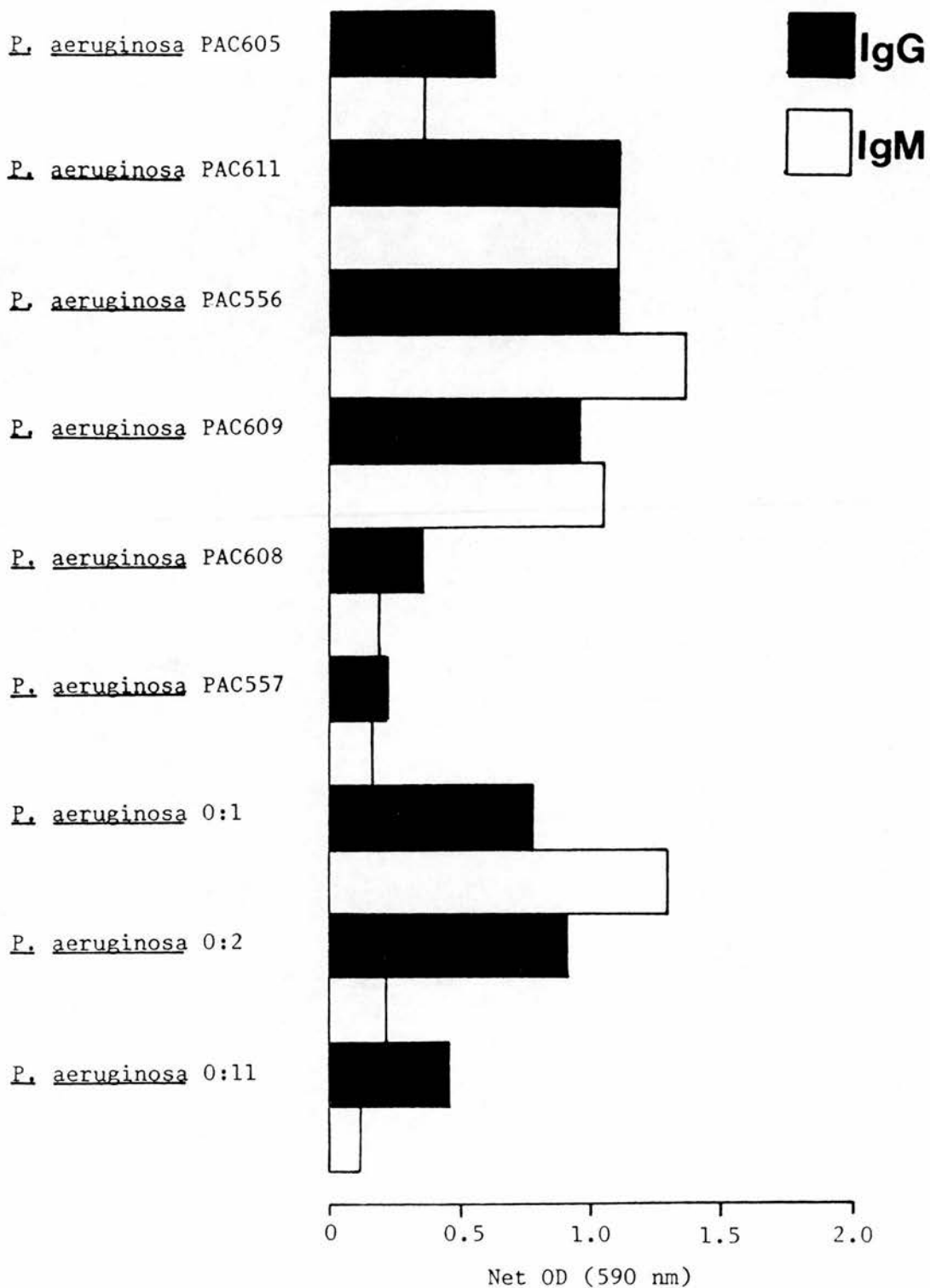


Figure 30. Serum anti-*P. aeruginosa* LPS antibodies in a female **BALB/c** mouse immunised with heat-killed cells of *P. aeruginosa* strains PAC608 and PAC605 which express only the core component of LPS. Antibodies were measured by ELISA with extracted LPS from 6 LPS defective mutants (PAC) and three LPS O-serotype *P. aeruginosa* strains.

directed towards these intermediate core structures were also observed in the ELISA screening of serum from the other four mice used in the study.

The mouse (Angel), for which data is shown, was used for the production of Mabs since it produced the strongest, most cross- reactive antibody response to the different *P. aeruginosa* LPS antigens.

3.2 PRIMARY AND SECONDARY SCREENING FOR ANTI-*P. AERUGINOSA* LPS MONOCLONAL ANTIBODIES

Mabs produced by the hybrids, and their resultant clones, were screened by ELISA. A primary screen consisting of three different LPS cocktails was used to gain an initial assessment of the LPS reactivity of antibodies present in hybrid and clone growth supernatants. Cocktail-A consisted of O-antigenic S-LPS from three serotype strains of *P. aeruginosa* (O:1, O:2 and O:11); cocktail-B consisted of core R-LPS from the six LPS defective mutants PAC605, PAC611, PAC556, PAC609, PAC608 and PAC557; cocktail-C (negative control) consisted of LPS from *S. typhimurium* 1542 and *E. coli* R1. Clones producing a strong antibody response (ELISA OD>1.0) directed to one or both of the *P. aeruginosa* LPS cocktails were selected and the antibodies subsequently tested in a secondary ELISA screen consisting of the individual LPS antigens which made up the LPS cocktails of the primary screen. The nature and extent of the reactivity of antibodies with the different LPS antigens was assessed. Results of some of the secondary screens are included in Figures 36-40 (see below).

3.3 SCREENING OF CROSS-REACTIVE ANTI-*P. AERUGINOSA* LPS MONOCLONAL ANTIBODIES

Based on results from the secondary ELISA screens, antibodies which cross reacted (ELISA OD>0.1) with all or at least seven out of the nine *P. aeruginosa* LPS antigens were selected for further study. Five Mabs were selected: Mabs 304.1.4, 360.7. 61.3.2, 73.5 and 334.4. These antibodies were subsequently

screened and characterised in ELISA against a comprehensive range of antigens, the results of which are shown in Figures 31-45.

The ELISA antigens used in the screens included: i) *P. aeruginosa* LPS antigens (incorporating those used in the secondary screens) including S-LPS from standard serotype strains; R-LPS from the PAC mutants and LPS from two non-mucoid *P. aeruginosa* strains isolated from patients with CF; ii) *P. aeruginosa* whole cell antigens including standard serotype strains O:1-O:16; core, LPS defective (PAC) mutants; and non-mucoid strains isolated from patients with CF; iii) PVA O:1-O:16; iv) whole cell antigens from other *Pseudomonas* spp as well as whole cells from other Gram-negative bacteria including *H. influenzae*, *H. parainfluenzae*, *E. coli*, *K. aerogenes* and *Pr. mirabilis*.

a) *P. aeruginosa* whole cell antigen ELISA screens

The five Mabs produced a positive response against all the *P. aeruginosa* whole cell antigens used in the screens (Figures 31-35). A strong reaction (ELISA OD > 1.0) against most of the whole cell antigens was particularly evident for Mabs 304.1.4 and 360.7 (Figures 31 and 32). All five Mabs tended to produce a lower response with whole cells from serotypes O:1 and O:4. This may either reflect the nature of the antibody binding site, or the coating of these whole cell antigens to the wells of the ELISA microtitre plate.

b) *P. aeruginosa* LPS and vaccine antigen ELISA screens

Confirmation of the cross-reactive nature of the five Mabs with all serotypes of *P. aeruginosa* was provided by the positive response of each Mab to the cell wall extracts of the vaccine antigens (PVA), serotypes O:1-O:16 (Figures 36-40). The strongest reactions were again observed for Mabs 304.1.4 and 360.7 (Figures 36 and 37).

WHOLE CELL ANTIGENS

Mab 304-1-4

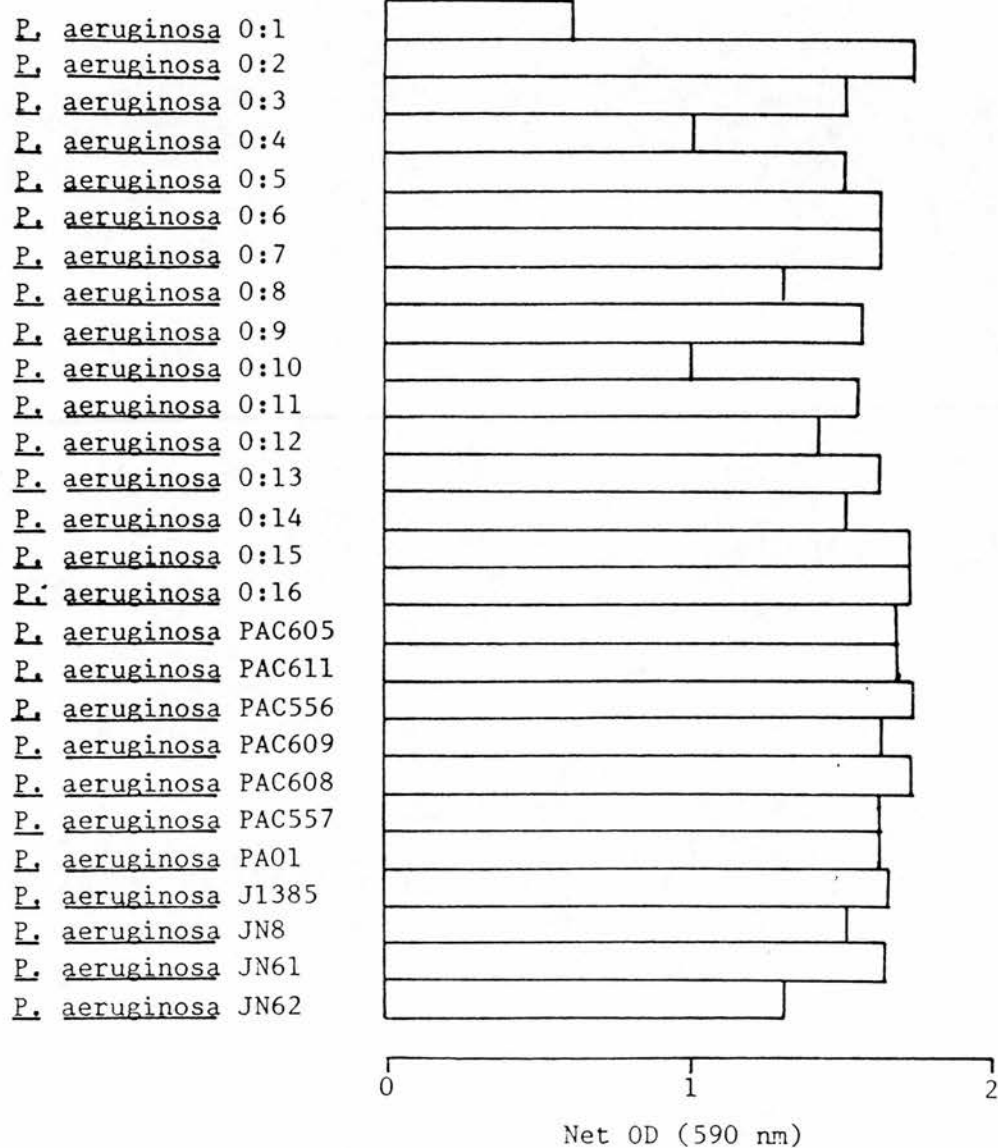


Figure 31. Reactivity in ELISA of Mab 304.1.4 against *P. aeruginosa* whole cell antigens.

WHOLE CELL ANTIGENS

Mab 360-7

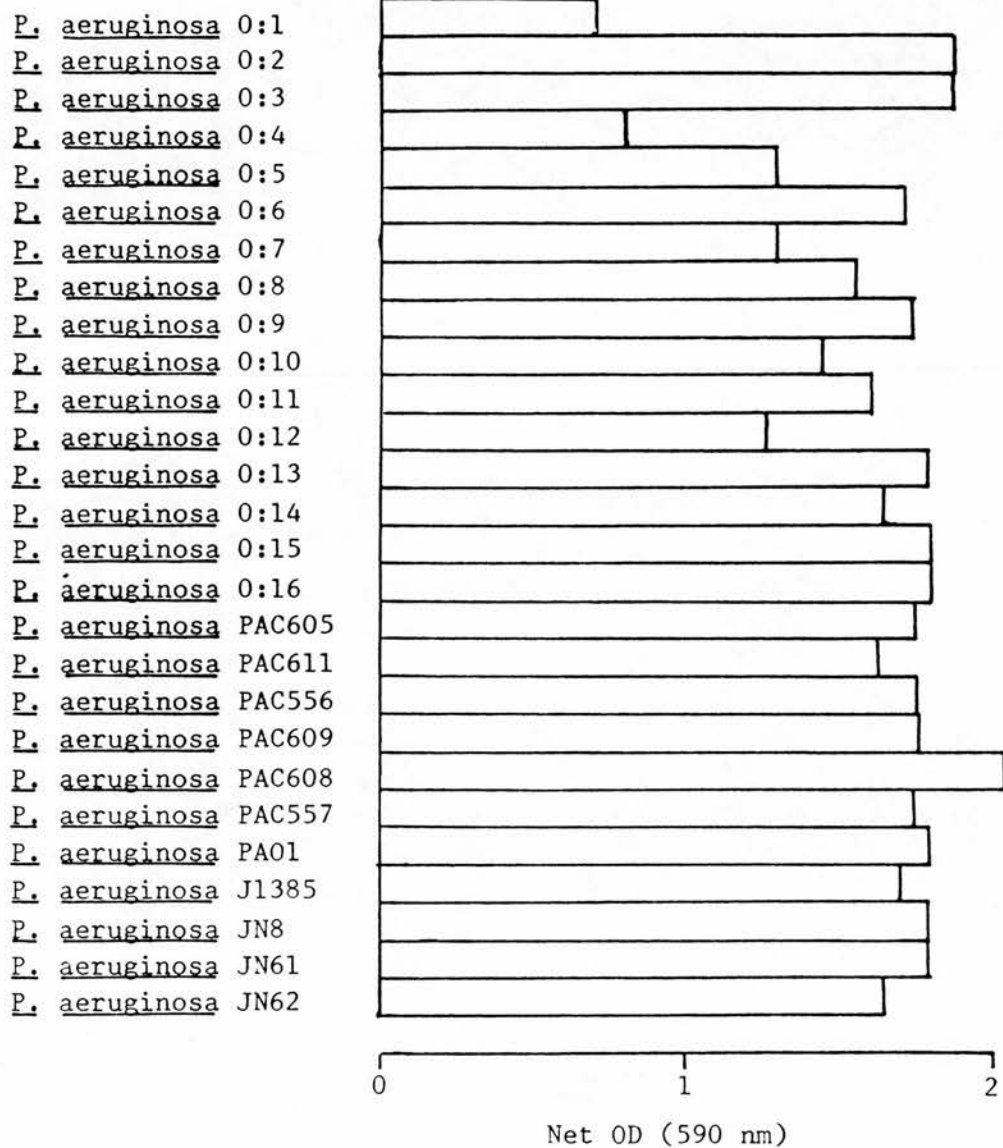


Figure 32. Reactivity in ELISA of Mab 360.7 against *P. aeruginosa* whole cell antigens.

WHOLE CELL ANTIGENS

Mab 61-3-2

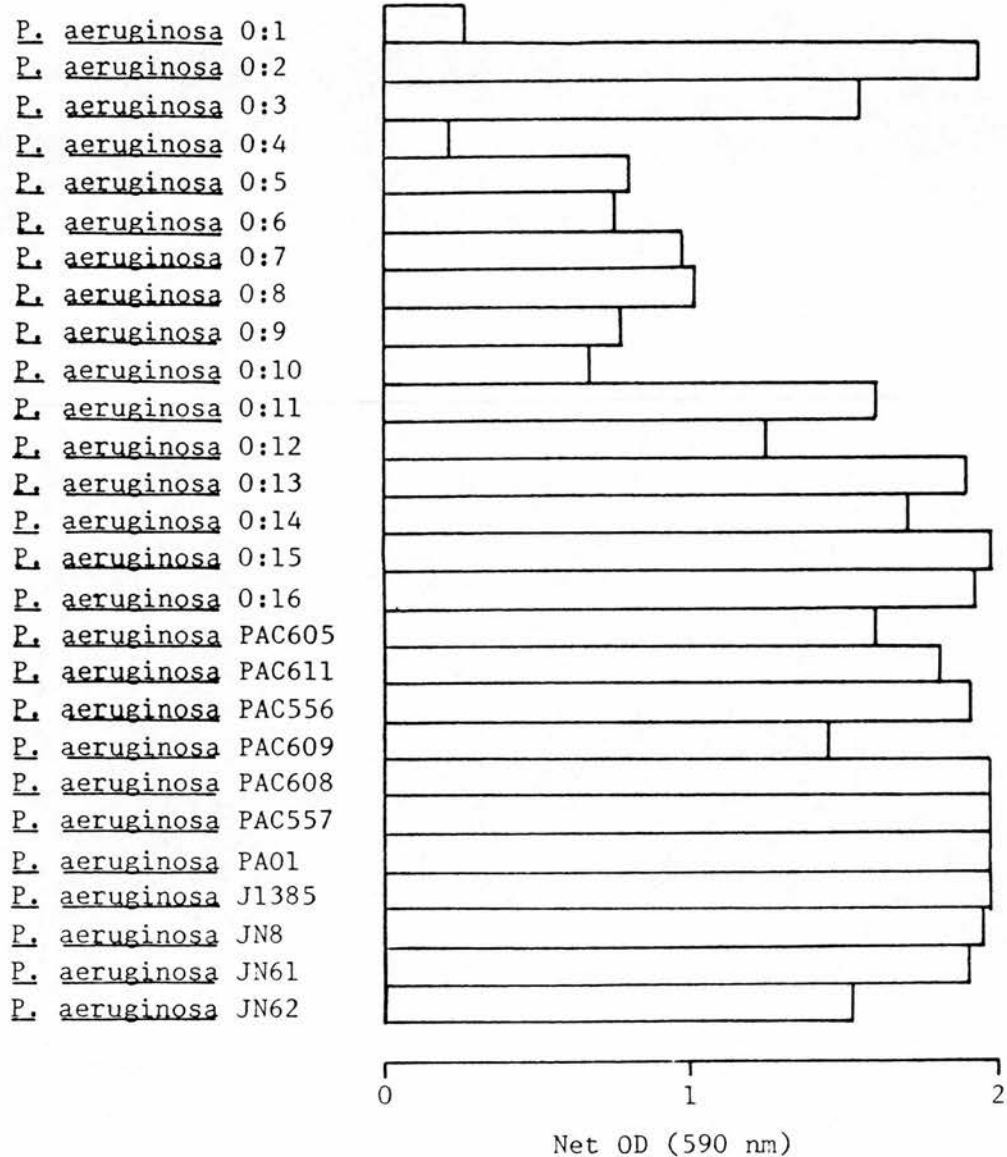


Figure 33. Reactivity in ELISA of Mab 61.3.2 against *P. aeruginosa* whole cell antigens.

WHOLE CELL ANTIGENS

Mab 73.5

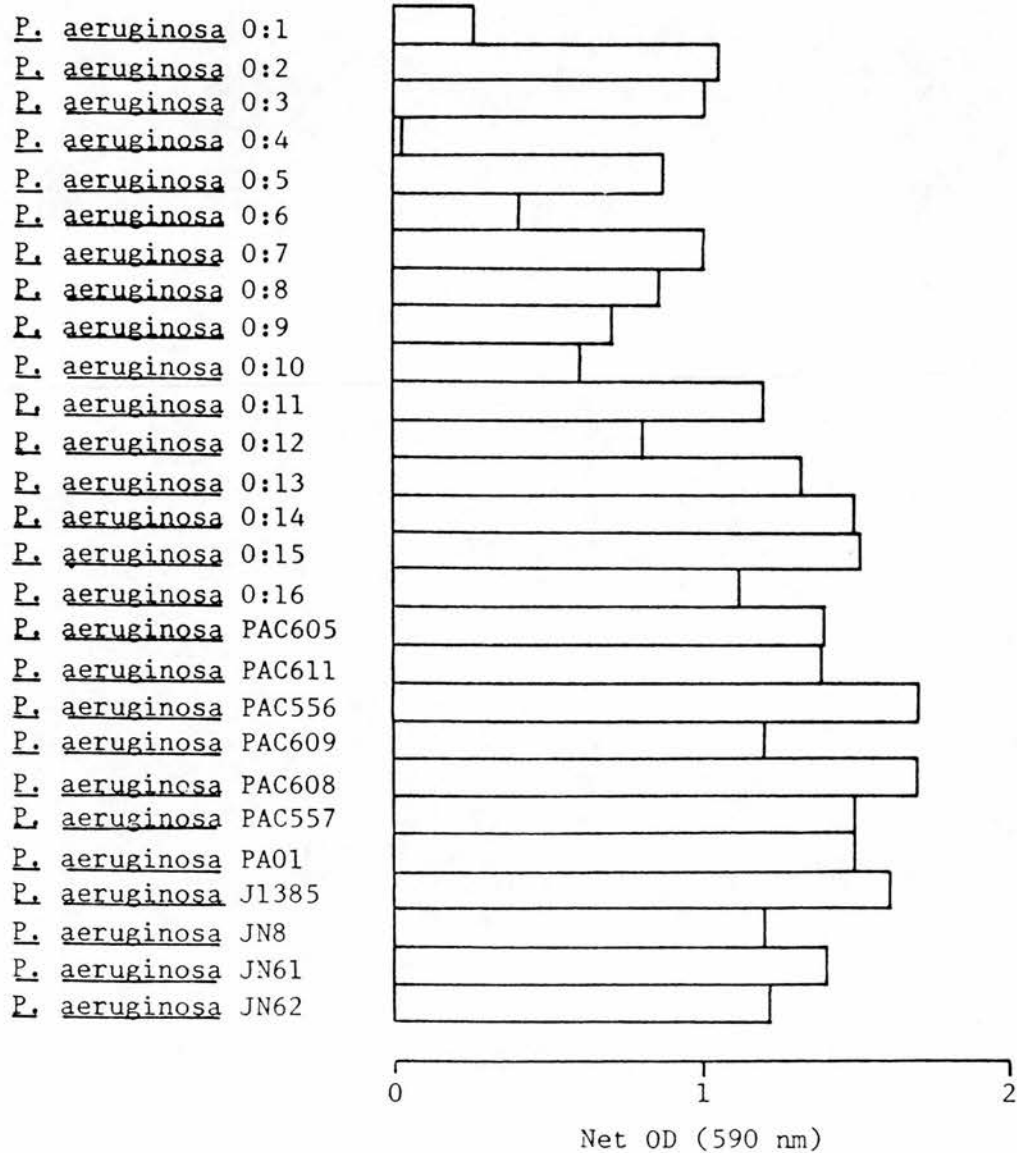


Figure 34. Reactivity in ELISA of Mab 73.5 against *P. aeruginosa* whole cell antigens.

WHOLE CELL ANTIGENS

Mab 334.4

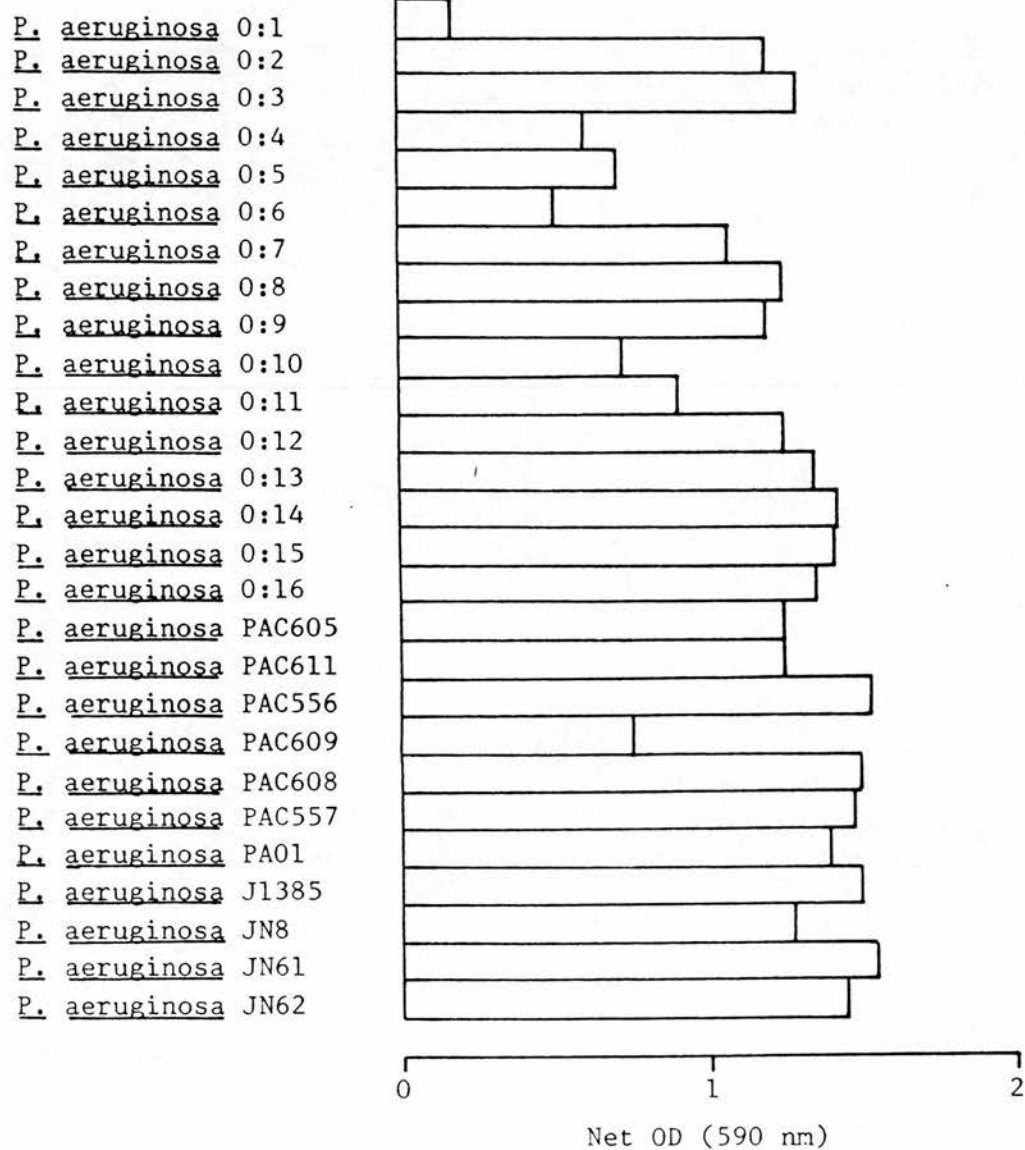


Figure 35. Reactivity in ELISA of Mab 334.4 against *P. aeruginosa* whole cell antigens.

The Mabs also reacted with each of the extracted *P. aeruginosa* LPS antigens: Mabs 304.1.4 and 360.7 generally showed the strongest reactions for each of the LPS antigens. Compared to the responses of the Mabs with whole cell and vaccine antigens, the absorbance readings obtained for the different LPS antigens tended to show greater variation. This was particularly apparent for Mabs 61.3.2, 73.5 and 334.4 (Figures 38-40). The response of the five Mabs to core R-LPS from the PAC mutants tended to be lower towards the shortest core structure, PAC605, and the complete core structure, PAC557, ie for each individual Mab, OD values were generally higher against the intermediate core structures (PAC611, PAC556, and PAC609). Unlike the reduced Mab responses to whole cells from *P. aeruginosa* serotype O:1, the responses to extracted LPS from serotype O:1 were generally higher and comparable to the reactions observed with the other LPS serotypes. The antibodies did not react with the enterobacterial LPS antigens from *E. coli* R1 and *S. typhimurium* 1542. The latter were chosen since potential reaction of the Mabs with enterobacterial LPS was most likely to be detected with these LPS antigens (Dr G.R. Barclay pers. comm.).

3.4 PSEUDOMONAS SPECIES AND OTHER GRAM-NEGATIVE WHOLE CELL ELISA SCREENS

The Mabs also showed reactions with whole cells from other *Pseudomonas* spp (Figures 41-45). All five Mabs reacted with the whole cells of *P. mendocina*, *P. fluorescens*, *P. pseudoalkaligenes*, *P. putida* and *P. stutzeri*; all the Mabs except 61.3.2 produced a positive reaction with *P. acidovorans* whilst only Mab 73.5 produced a detectable reaction with *P. testosteroni*.

The Mabs were also screened against the whole cells of ten individual strains of *P. cepacia* and seven strains of *P. maltophilia*; these two *Pseudomonas* species are the most common pseudomonads, after *P. aeruginosa*, to be isolated from the respiratory secretions of patients with CF. Mab 304.1.4 (Figure 41) failed to show a detectable reaction with any of the *P. cepacia* or *P. maltophilia* strains,

ELISA ANTIGENS

Mab 304.1.4

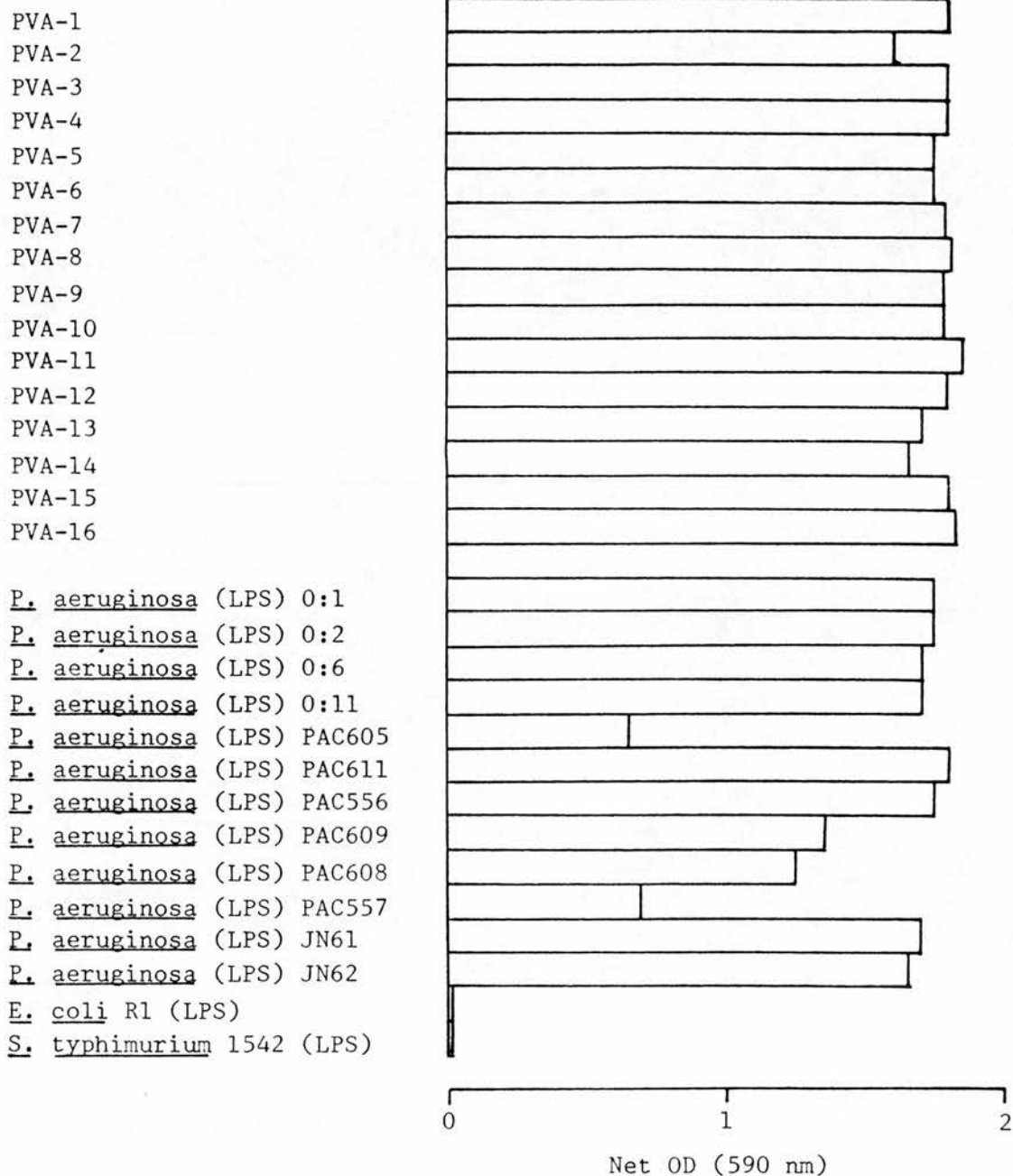


Figure 36. Reactivity in ELISA of Mab 304.1.4 against i) *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1 - PVA-16); ii) extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O:1-O:11) and R-LPS (PAC605-PAC557); iii) LPS from two *P. aeruginosa* strains (JN61 and JN62) from patients with CF, and iv) LPS from two *Enterobacteriaceae* (negative controls).

ELISA ANTIGENS

Mab 360·7

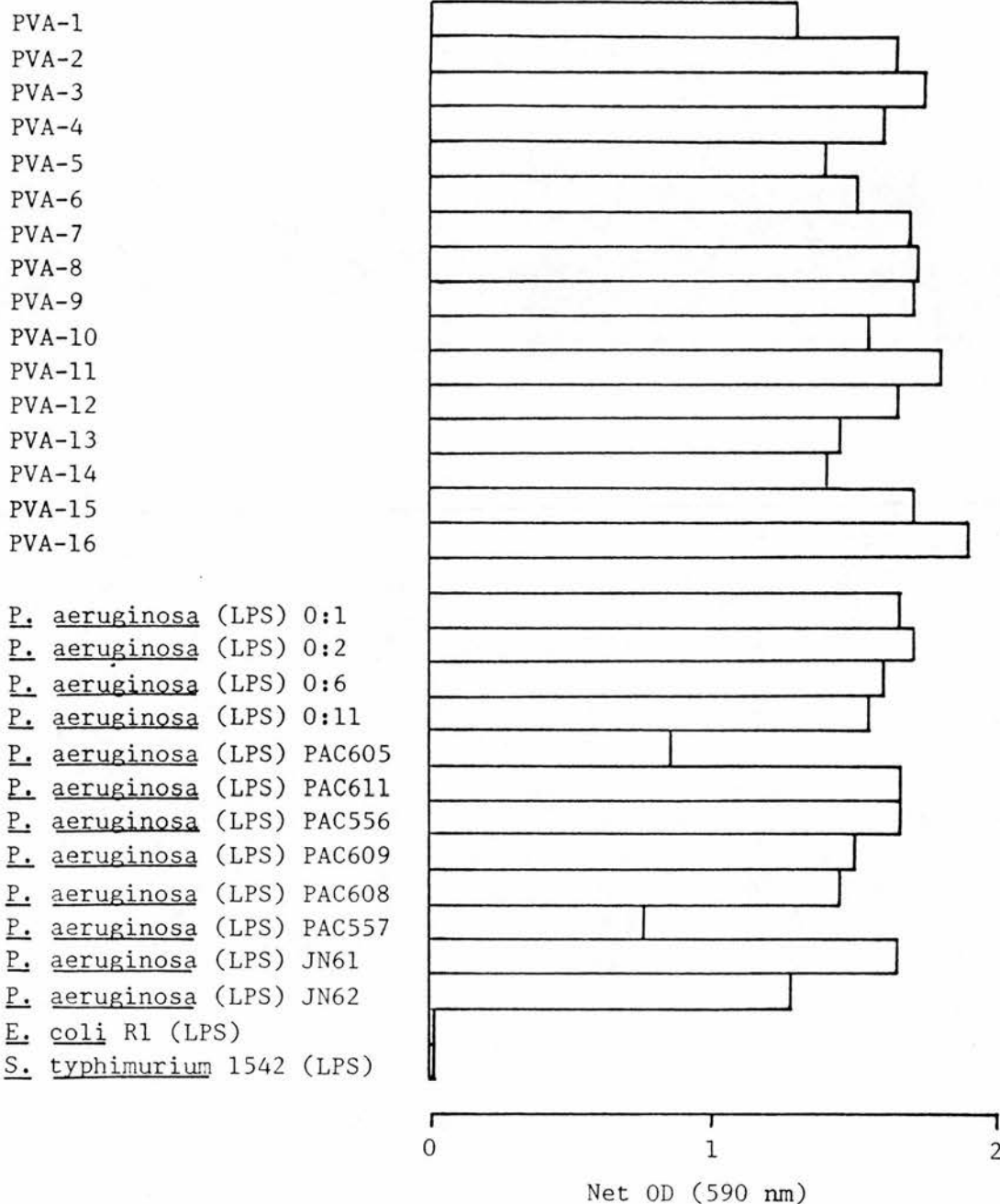


Figure 37. Reactivity in ELISA of Mab 360.7 against i) *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1 - PVA-16); ii) extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O:1-O:11) and R-LPS (PAC605-PAC557); iii) LPS from two *P. aeruginosa* strains (JN61 and JN62) from patients with CF, and iv) LPS from two *Enterobacteriaceae* (negative controls).

ELISA ANTIGENS

Mab 61.3.2

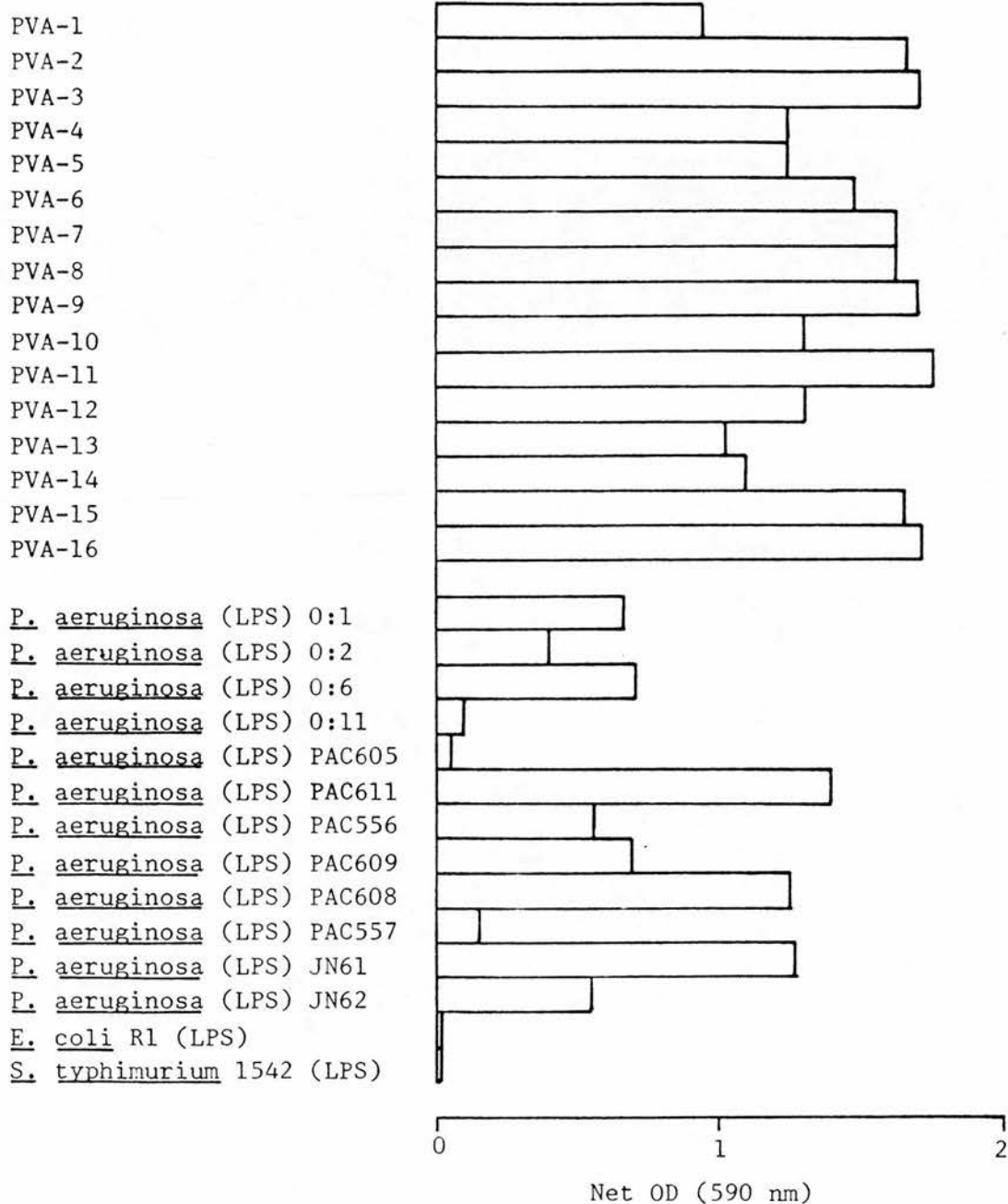


Figure 38. Reactivity in ELISA of Mab 61.3.2 against i) *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1 - PVA-16); ii) extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O:1-O:11) and R-LPS (PAC605-PAC557), iii) LPS from two *P. aeruginosa* strains (JN61 and JN62) from patients with CF, and iv) LPS from two *Enterobacteriaceae* (negative controls).

ELISA ANTIGENS

Mab 73.5

PVA-1
PVA-2
PVA-3
PVA-4
PVA-5
PVA-6
PVA-7
PVA-8
PVA-9
PVA-10
PVA-11
PVA-12
PVA-13
PVA-14
PVA-15
PVA-16

P. aeruginosa (LPS) O:1
P. aeruginosa (LPS) O:2
P. aeruginosa (LPS) O:6
P. aeruginosa (LPS) O:11
P. aeruginosa (LPS) PAC605
P. aeruginosa (LPS) PAC611
P. aeruginosa (LPS) PAC556
P. aeruginosa (LPS) PAC609
P. aeruginosa (LPS) PAC608
P. aeruginosa (LPS) PAC557
P. aeruginosa (LPS) JN61
P. aeruginosa (LPS) JN62
E. coli R1 (LPS)
S. typhimurium 1542 (LPS)

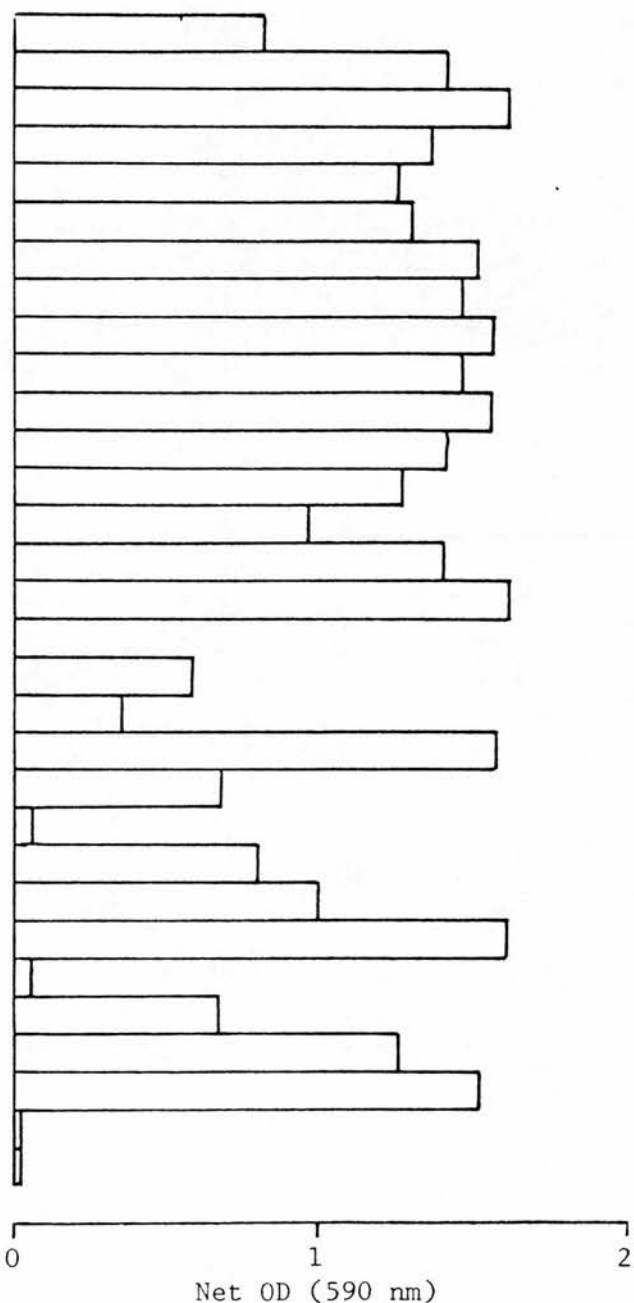


Figure 39. Reactivity in ELISA of Mab 73.5 against i) *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1 - PVA-16), ii) extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O:1-O:11) and R-LPS (PAC605-PAC557); iii) LPS from two *P. aeruginosa* strains (JN61 and JN62) isolated from patients with CF, and iv) LPS from two *Enterobacteriaceae* (negative controls).

ELISA ANTIGENS

PVA-1
PVA-2
PVA-3
PVA-4
PVA-5
PVA-6
PVA-7
PVA-8
PVA-9
PVA-10
PVA-11
PVA-12
PVA-13
PVA-14
PVA-15
PVA-16

P. aeruginosa (LPS) 0:1
P. aeruginosa (LPS) 0:2
P. aeruginosa (LPS) 0:6
P. aeruginosa (LPS) 0:11
P. aeruginosa (LPS) PAC605
P. aeruginosa (LPS) PAC611
P. aeruginosa (LPS) PAC556
P. aeruginosa (LPS) PAC609
P. aeruginosa (LPS) PAC608
P. aeruginosa (LPS) PAC557
P. aeruginosa (LPS) JN61
P. aeruginosa (LPS) JN62
E. coli R1 (LPS)
S. typhimurium 1542 (LPS)

Mab 334.4

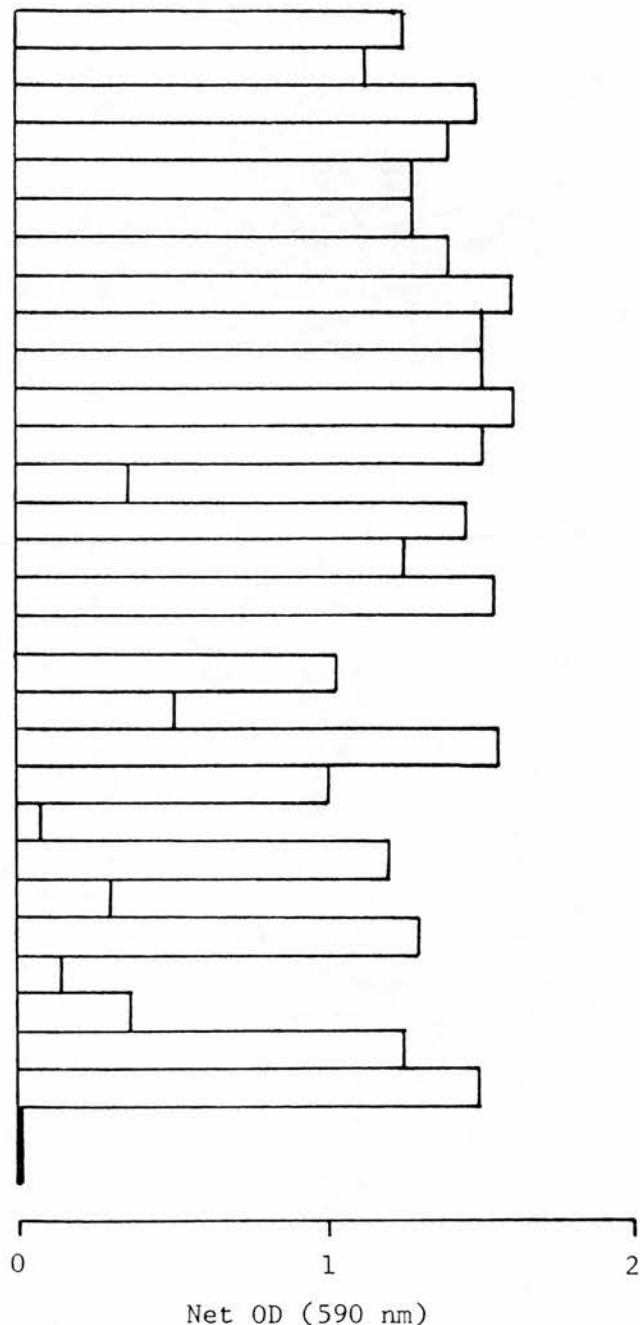


Figure 40. Reactivity in ELISA of Mab 334.4 against i) *P. aeruginosa* S-LPS O-serotype vaccine components (PVA1 - PVA16); ii) extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O:1-O:11) and R-LPS (PAC605-PAC557); iii) LPS from two *P. aeruginosa* strains (JN61 and JN62) isolated from patients with CF, and iv) LPS from two *Enterobacteriaceae* (negative controls).

whilst Mabs 73.5 (Figure 44) and 334.4 (Figure 45) produced a weak reaction with one of the *P. maltophilia* strains (different in each case). Mabs 360.7 (Figure 42) and 61.3.2 (Figure 43) also failed to produce a positive reaction with any of the *P. cepacia* strains. However, these Mabs reacted weakly with some of the *P. maltophilia* strains; Mab 360.7 produced weak (all ODs <0.25) reactions with *P. maltophilia* strains J115, J118, J122 and J135, whilst Mab 61.3.2 reacted weakly (all ODs <0.30) with five out of the seven *P. maltophilia* strains.

In most cases the five Mabs failed to react with whole cells of *H. influenzae* and *H. parainfluenzae* (Figures 41-45). Only Mabs 360.7 and 61.3.2 produced detectable reaction with any of these organisms; both reacted very weakly (ELISA OD <0.08) with one of the *H. influenzae* strains and the *H. parainfluenzae* strain. None of the Mabs reacted with the whole cells of *E. coli*, *K. aerogenes*, and *Pr. mirabilis*.

3.5 IMMUNOBLOT ANALYSIS OF MONOCLONAL ANTIBODIES AGAINST LPS

The ELISA studies showed that the Mabs were able to react with all the serotypes of *P. aeruginosa* as well as with LPS deficient mutants expressing only core R-LPS. Since the Mabs were originally produced from spleen cells derived from a mouse vaccinated with heat killed cells from core R-LPS mutants, these results would indicate that the Mabs recognise the common core component of *P. aeruginosa* LPS. In order to confirm and further investigate the LPS binding site of each of the Mabs, immunoblot assays against LPS from proteinase K digests of *P. aeruginosa* cells were performed. The immunoblots of the five Mabs against S-LPS of *P. aeruginosa* serotype strains, and core R-LPS of *P. aeruginosa* LPS defective mutants, are shown in Figures 46-48.

WHOLE CELL ANTIGENS

Mab 304.1.4

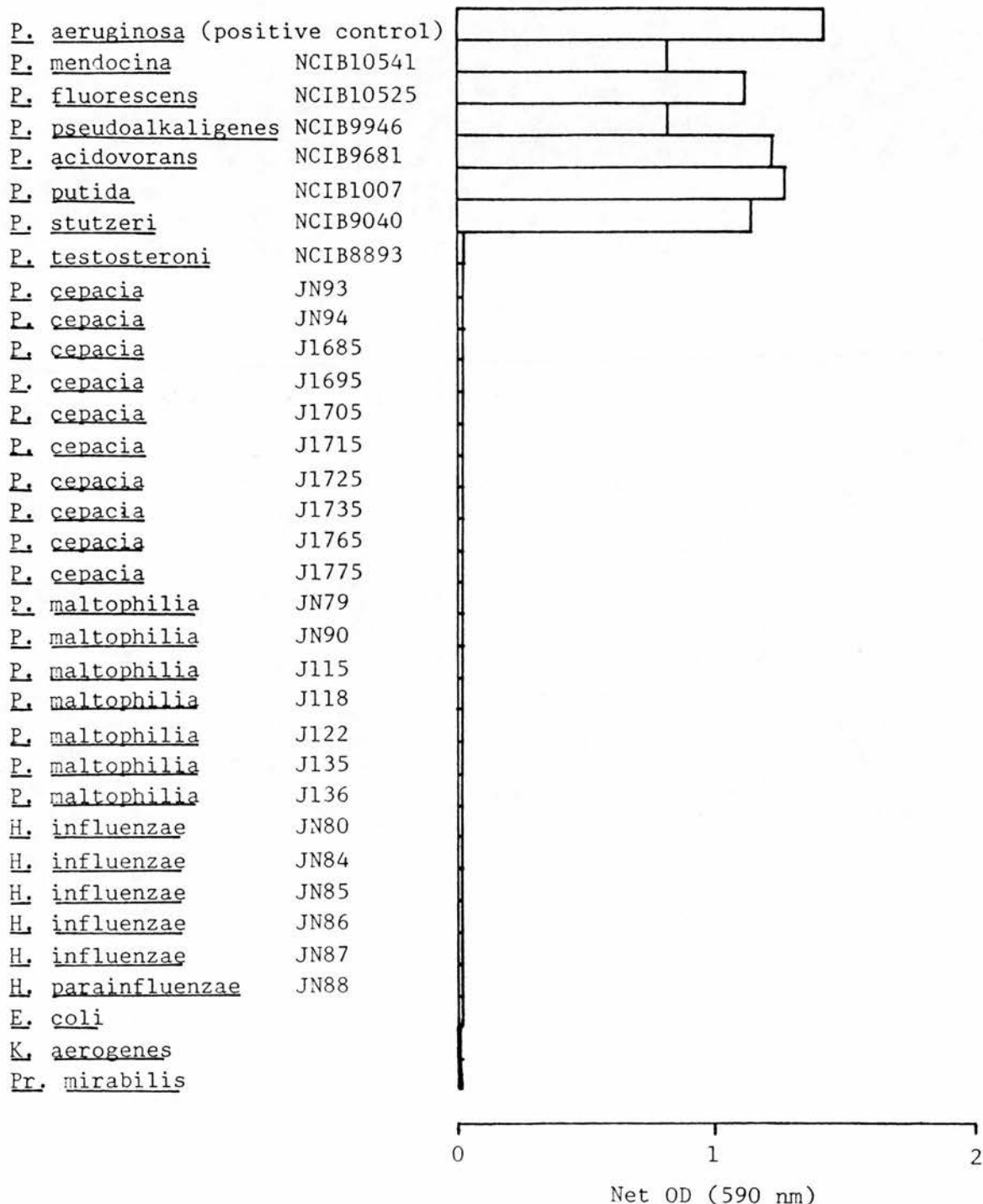


Figure 41. Reactivity in ELISA of Mab 304.1.4 against whole cell antigens.

WHOLE CELL ANTIGENS

Mab 360.7

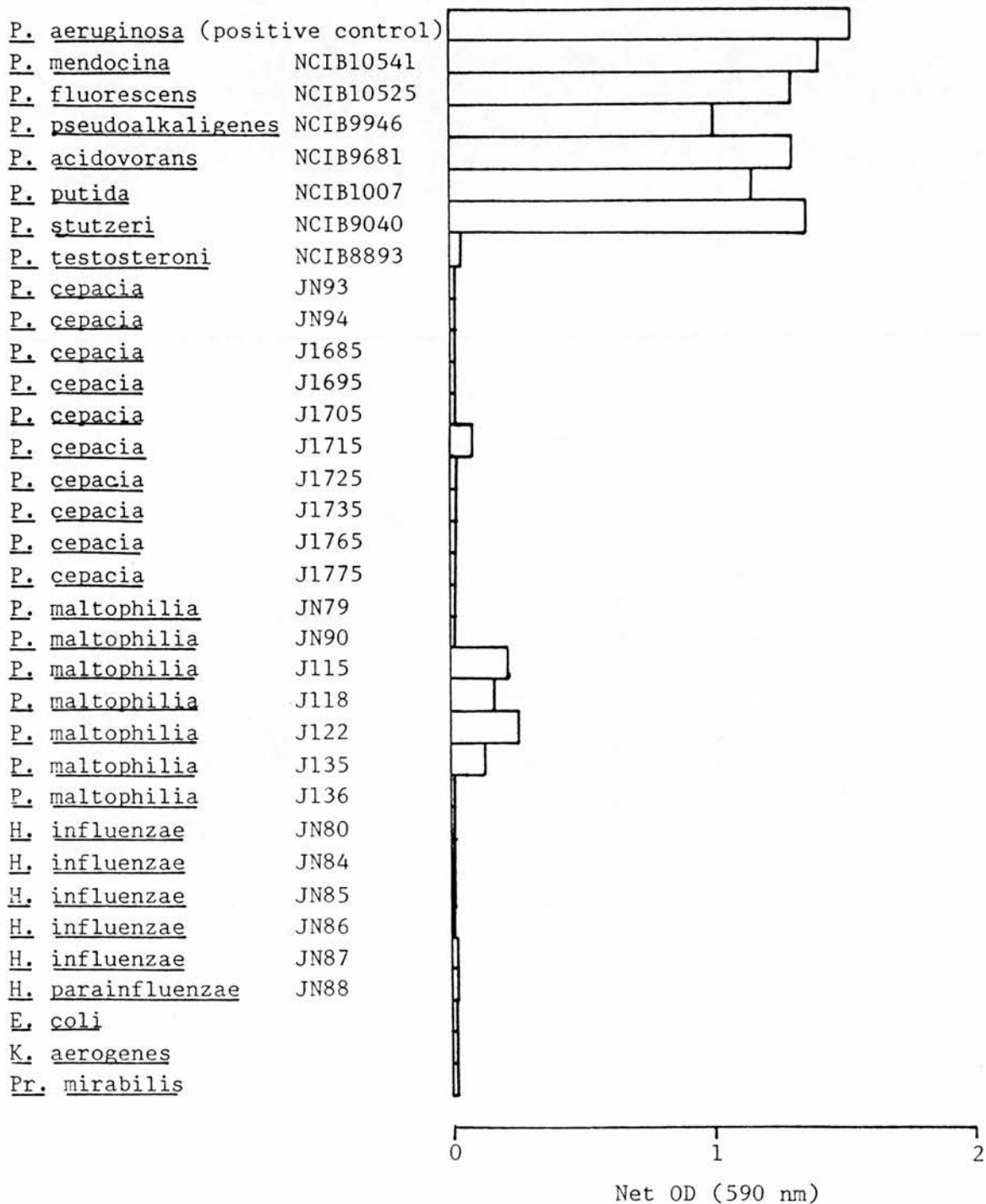


Figure 42. Reactivity in ELISA of Mab 360.7 against whole cell antigens.

WHOLE CELL ANTIGENS

Mab 61-3-2

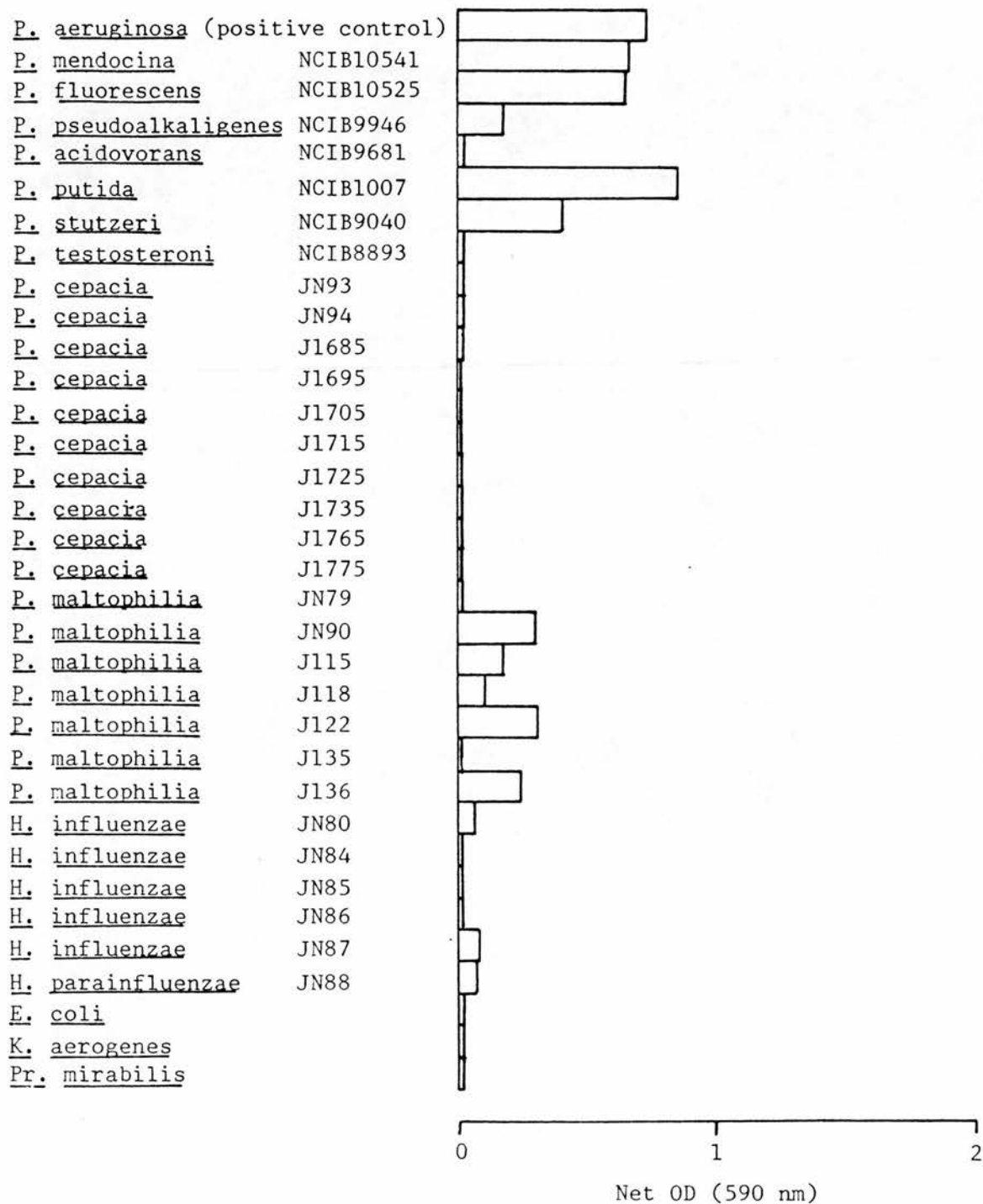


Figure 43. Reactivity in ELISA of Mab 61.3.2 against whole cell antigens.

WHOLE CELL ANTIGENS

Mab 73-5

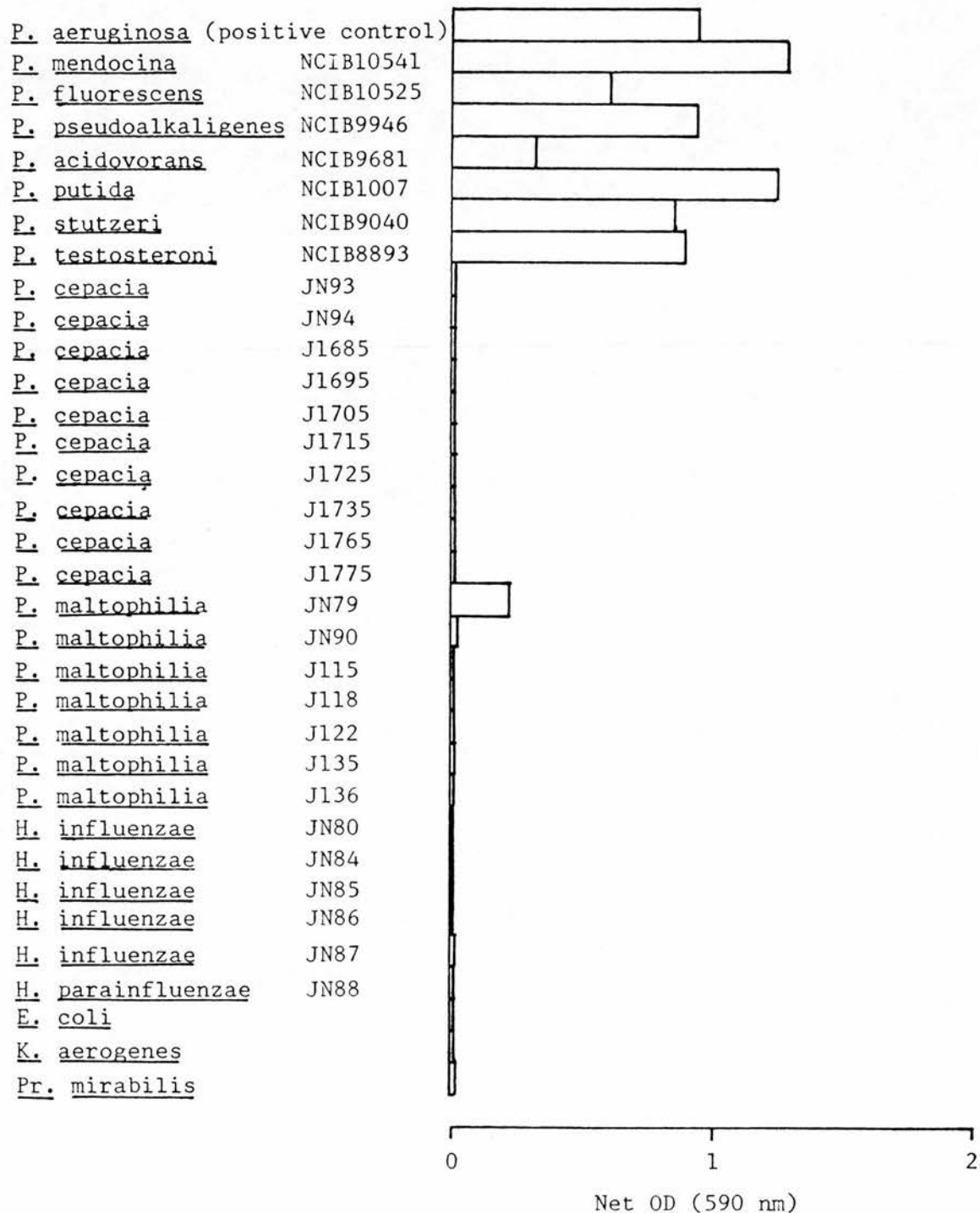


Figure 44. Reactivity in ELISA of Mab 73.5 against whole cell antigens.

WHOLE CELL ANTIGENS

Mab 334.4

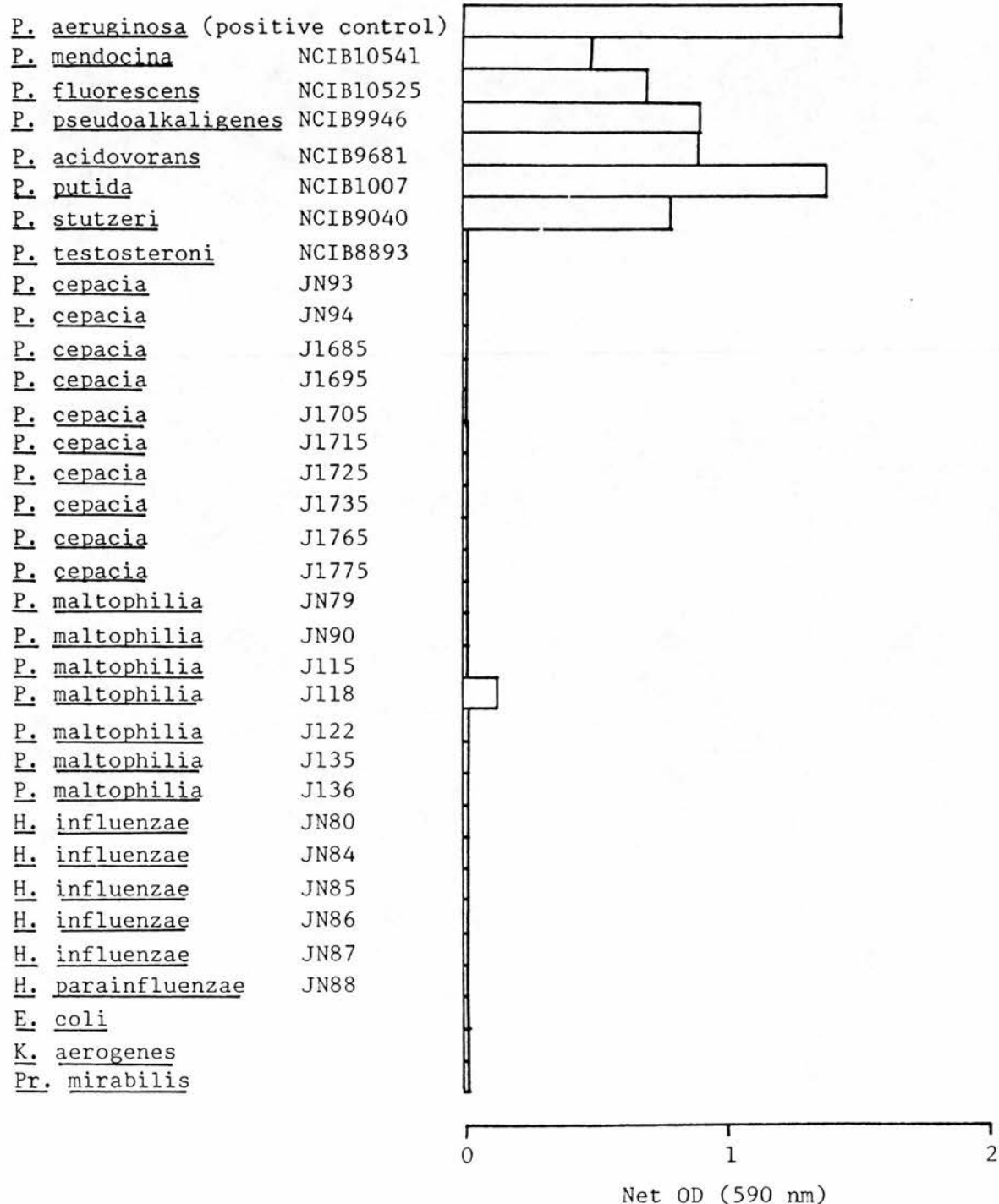


Figure 45. Reactivity in ELISA of Mab 334.4 against whole cell antigens.

The immunoblots of Mabs 304.1.4 and 360.7 are shown in Figure 46. A positive reactive band corresponding to the low molecular weight core LPS of each of the *P. aeruginosa* LPS preparations analysed was observed. The weakest responses were against the core LPS regions of serotypes O:9 (Track 9) and O:11 (Track 11) and with R-LPS core of PAC605 (Track 16). The apparent absence of bands corresponding to high molecular weight LPS of serotype strains indicated that the Mabs were unable to recognise core LPS substituted with O-antigenic side chains. However, a very faint ladder pattern corresponding to the high molecular weight O-antigenic LPS of serotype O:6 (Track 6) was observed in both cases, especially in the case of Mab 360.7.

The immunoblots obtained with Mabs 61.3.2, 73.5 and 334.4 (Figures 47 and 48) were all very much weaker than the immunoblots obtained for Mabs 304.1.4 and 360.7 (Figure 46). Indeed, immunoblots with the former antibodies were only obtained using the Mab concentrates prepared as described in the Materials and Methods. The concentrates were twenty-fold concentrations of Mab containing supernatants.

Mab 61.3.2 reacted weakly with most of the unsubstituted core regions of the different LPS preparations (Figure 47). The strongest reactions were generally those observed against the core regions of the R-LPS mutants PAC609, PAC556, PAC557 and PAC608 (Tracks 17-20 respectively). However, a reaction was not obtained against the two roughest PAC mutants, *P. aeruginosa* strains PAC605 and PAC611 (Tracks 15 and 16). In the ELISA studies Mab 61.3.2 reacted with whole cell and LPS antigens of all the PAC mutants, although only weakly with PAC605 LPS. Similarly, in the immunoblot obtained with Mab 73.5 (Figure 47) a reaction with most of the core components of the different LPS preparations was observed. However, as with Mab 61.3.2, a reaction with PAC605 or PAC611 (Tracks 15 and 16) was not detected.

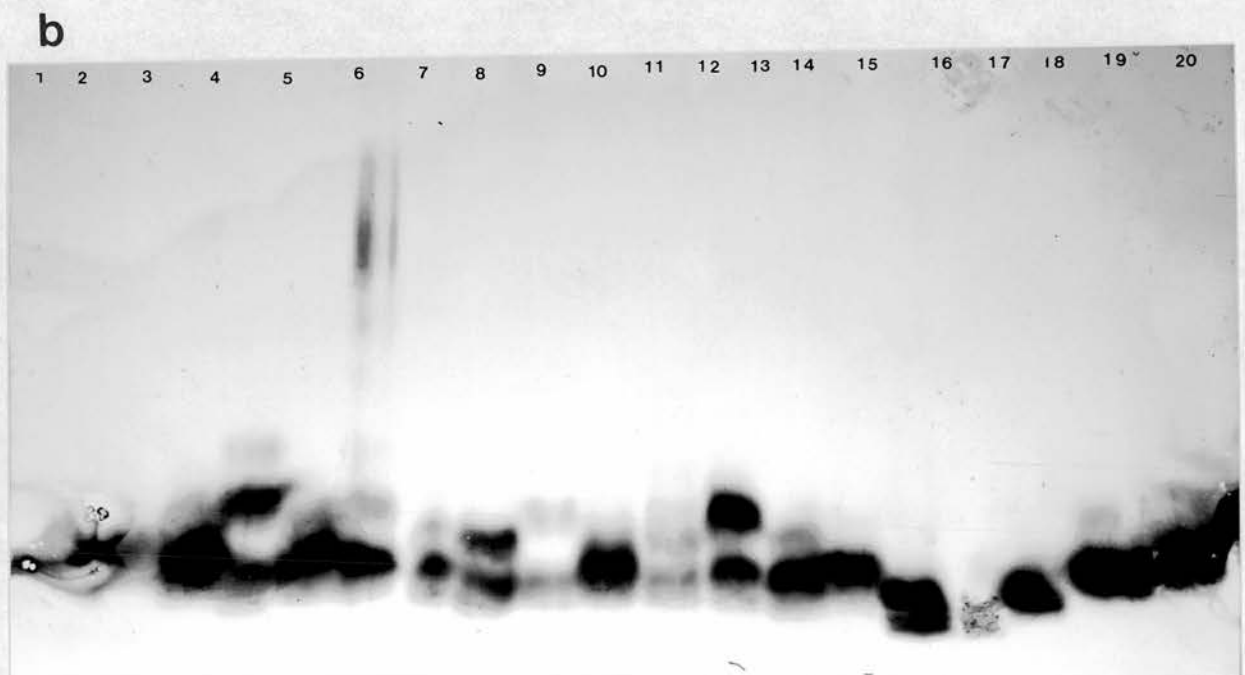
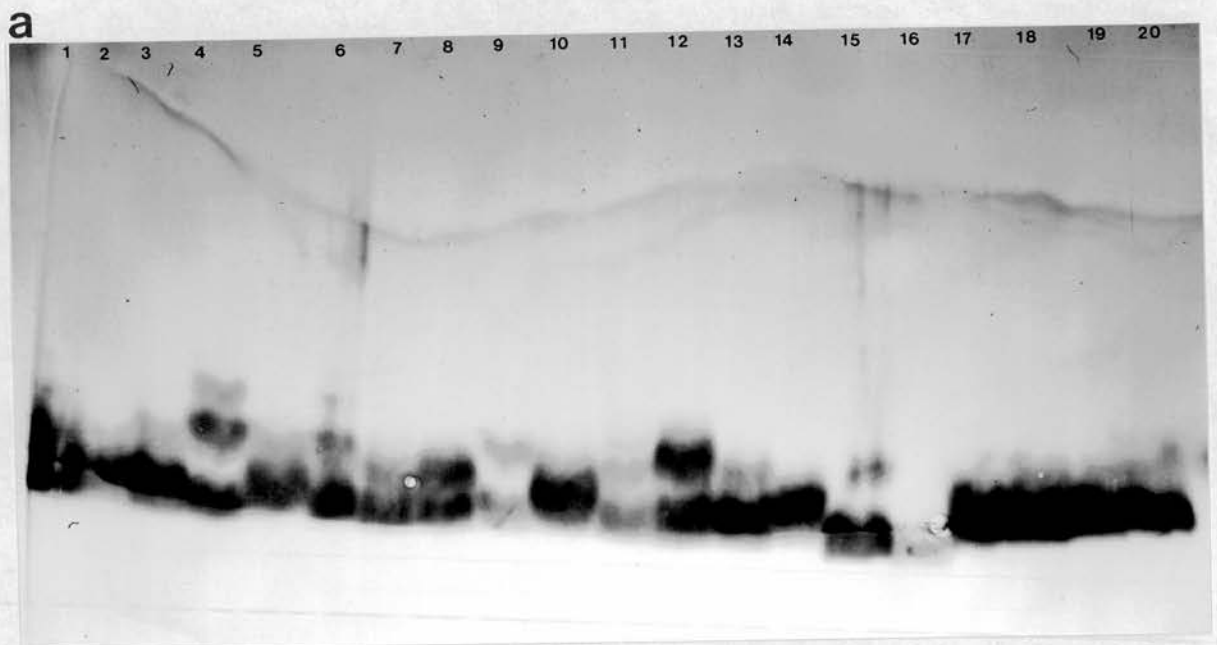


Figure 46. Immunoblot of proteinase K whole cell digests of 20 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with Mabs a) 304.1.4 and b) 360.7 (supernatant 1:5). The immunoblot profiles against *P. aeruginosa* serotype strains O:1, O:2, O:3, O:4, O:5, O:6, O:7, O:8, O:9, O:10, O:11, O:12, O:13, O:15 and *P. aeruginosa* LPS defective mutants PAC611, PAC605, PAC556, PAC609, PAC557 and PAC608 are shown in Tracks 1-20 respectively.

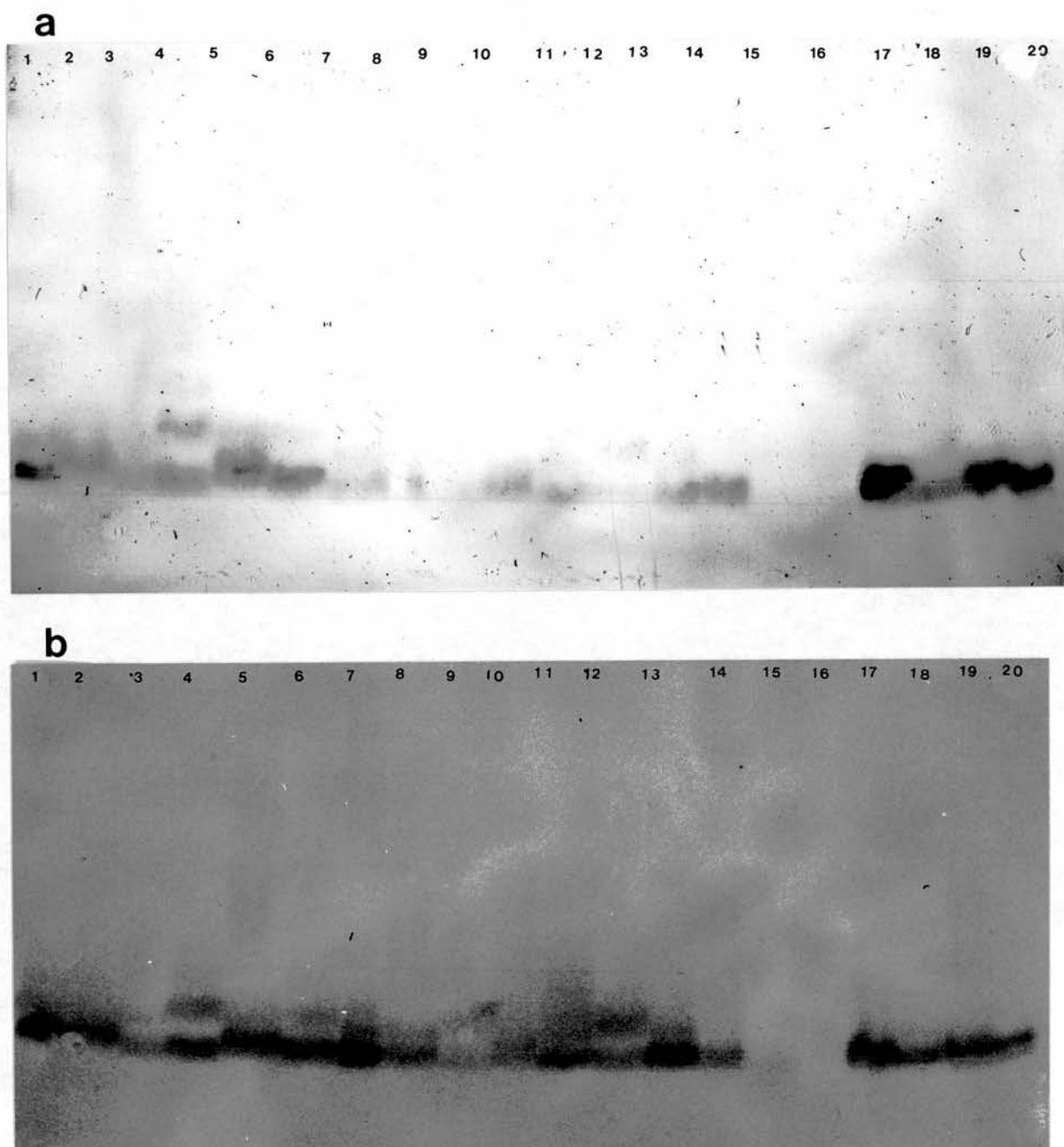


Figure 47. Immunoblots of proteinase K whole cell digests of 20 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with Mabs a) 61.3.2 and b) 73.5 (concentrate 1:5). The immunoblot profiles against *P. aeruginosa* serotype strains O:1, O:2, O:3, O:4, O:5, O:6, O:7, O:8, O:9, O:10, O:11, O:12, O:13, O:15 and *P. aeruginosa* LPS defective mutants PAC611, PAC605, PAC556, PAC609, PAC557, and PAC608 are shown in Tracks 1-20 respectively.

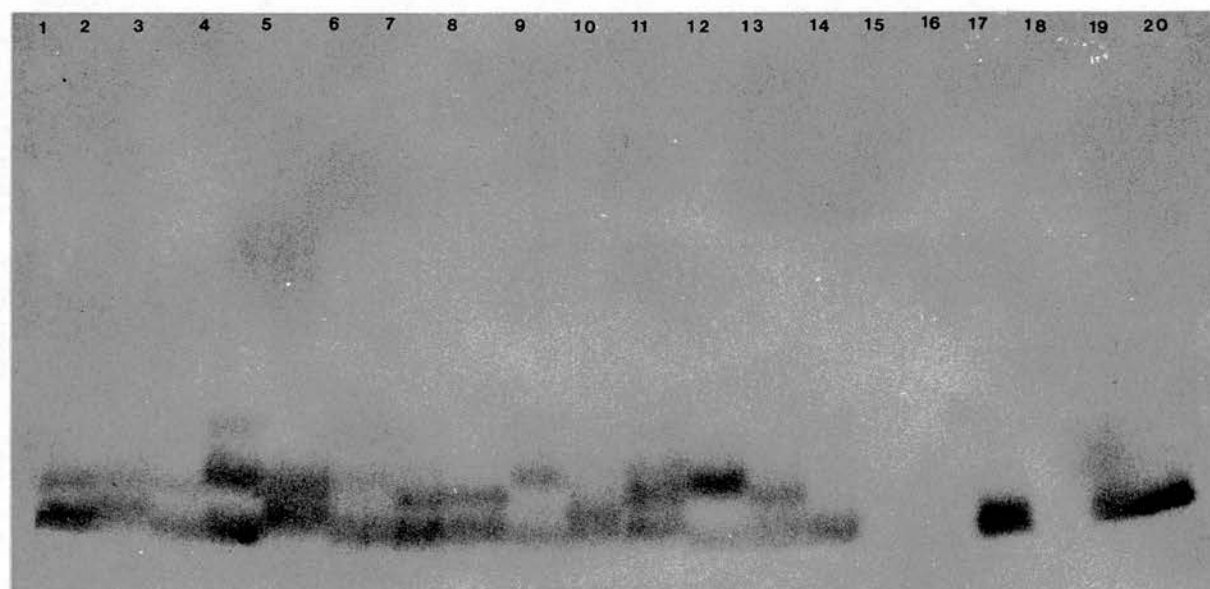


Figure 48. Immunoblot of proteinase K whole cell digests of 20 *P. aeruginosa* strains separated by SDS-PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with Mab 334.4 (concentrate 1:5). The immunoblot profiles against *P. aeruginosa* serotype strains O:1, O:2, O:3, O:4, O:5, O:6, O:7, O:8, O:9, O:10, O:11, O:12, O:13, O:15 and *P. aeruginosa* LPS defective mutants PAC611, PAC605, PAC556, PAC609, PAC557 and PAC608 are shown in Tracks 1-20 respectively.

The immunoblot obtained for Mab 334.4 (Figure 48) showed weak reactions with the unsubstituted core LPS of all serotype strains of *P. aeruginosa* (Tracks 1-14). However, reactions were observed with only three out of the six core R-LPS mutants, not being detected against PAC605, PAC611 and PAC609 (Tracks 15, 16 and 18 respectively). Except for the weak reaction of Mab 334.4 with PAC605 LPS, these immunoblot profiles were not reflected by the corresponding ELISA results.

Immunoblotting was also performed with Mabs 304.1.4 and 360.7 against LPS (prepared by proteinase K digestion of whole cells) from non-mucoid and mucoid *P. aeruginosa* strains isolated from patients with CF. The silver stained LPS profiles of each strain are shown in Figure 49. The two Mabs gave a strong positive band corresponding to the low molecular, unsubstituted core region of each LPS preparation (Figure 50a & b). The reaction of Mab 360.7 was particularly strong. In addition Mab 360.7 reacted very weakly with the high molecular weight LPS bands (ie core substituted with O-antigen LPS) of non-mucoid strain JN61 (serotype O:6) in Track 2 and mucoid strain JN10 (non-typable) in Track 8. These bands are not evident in the photograph.

3.6 TYPING AND ANALYSIS OF MONOCLONAL ANTIBODY PREPARATIONS

The Mabs were typed and classified as:

Mab 304.1.4	=	IgG2b
Mab 360.7	=	IgG2b
Mab 61.3.2	=	IgG3
Mab 73.5	=	IgG1
Mab 334.4	=	IgG3.

Mabs in bulk growth supernatants were concentrated twenty-fold and partially purified using a tangential-flow filtration system. The presence of antibody in the retentate and absence in the filtrate was checked by ELISA. The

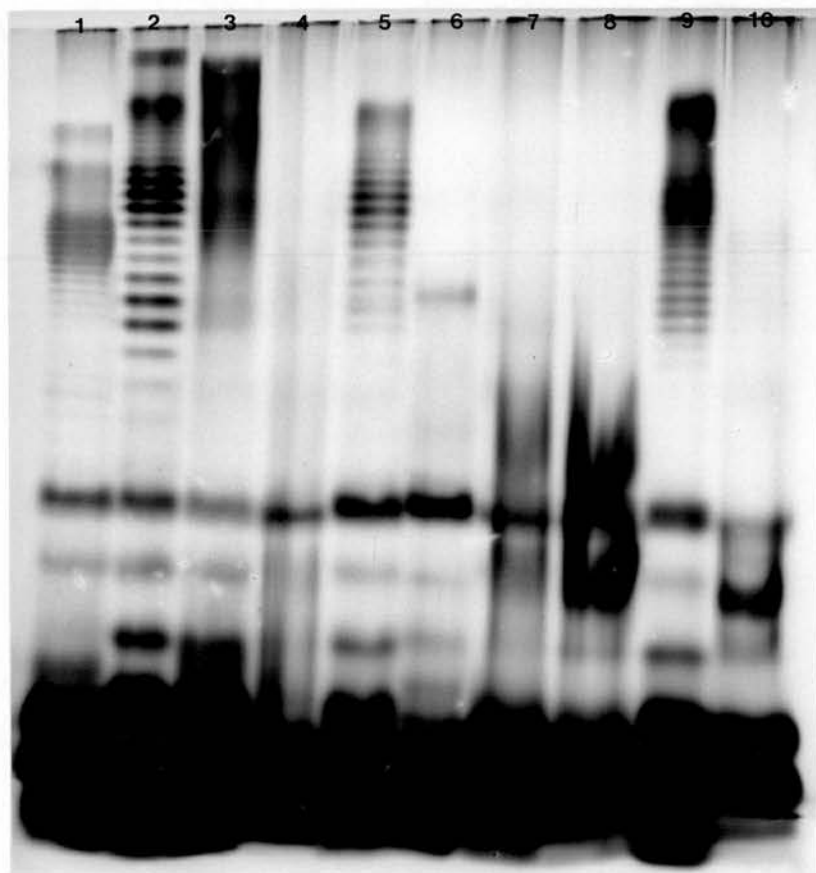


Figure 49. Silver-stained SDS-polyacrylamide gel of proteinase K- digested whole cell lysates from *P. aeruginosa* isolates from CF lung infection. The profiles of *P. aeruginosa* strains JN62 (non-mucoid, serotype O:1); JN61 (non-mucoid, serotype O:6); JN8 (non-mucoid, serotype O:6), JN28 (non-mucoid, NT); JN45 (non-mucoid, NT); JN9 (non-mucoid, NT); JN49 (non-mucoid, NT); JN50 (mucoid, NT), JN10 (mucoid NT) and JN56 (mucoid, NT) are shown in Tracks 1-10 respectively.

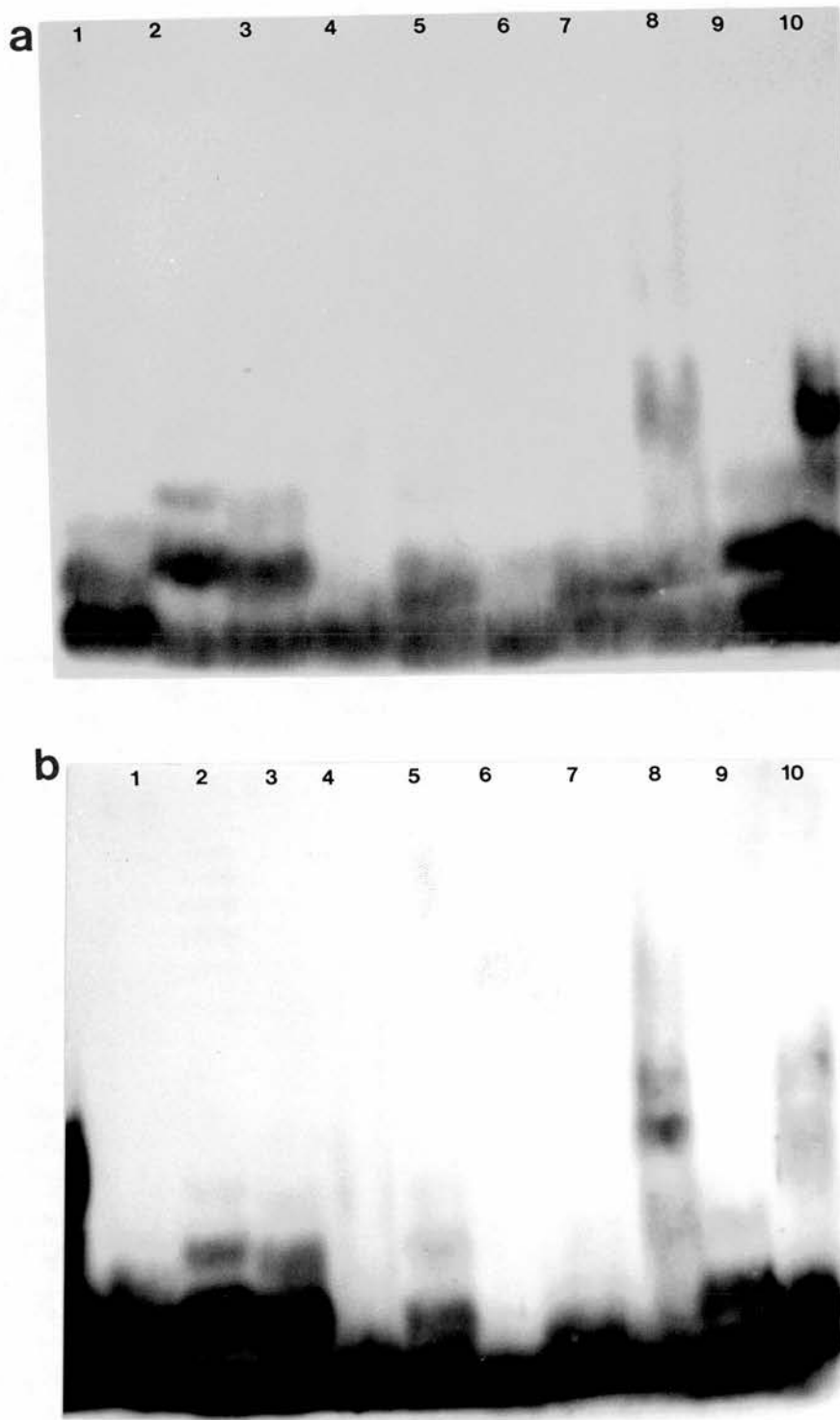


Figure 50. Immunoblots of proteinase K whole cell digests of 10 *P. aeruginosa* strains (isolated from patients with CF) separated by SDS- PAGE (14% w/v acrylamide) followed by transfer to NIC paper and probed with Mabs a) 304.1.4 and b) 360.7 (supernatants 1:5). The immunoblot profiles against *P. aeruginosa* strains JN62 (non-mucoid, serotype O:1); JN61 (non-mucoid, serotype O:6); JN8 (non-mucoid, serotype O:6); JN28 (non-mucoid, NT); JN45 (non-mucoid, NT) JN9 (non-mucoid, NT); JN49 (non-mucoid, NT); JN50 (mucoid, NT); JN10 (mucoid, NT) and JN56 (mucoid, NT) are shown in Tracks 1-10 respectively.

immunoglobulins in each concentrate were further purified by ammonium sulphate precipitation. The final preparations were titred in ELISA in order to check for the presence of antibody. The Mab preparations before and after ammonium sulphate precipitation were analysed by agarose gel electrophoresis and the Coomassie blue stained profiles are shown in Figure 51a. Ammonium sulphate precipitation of the Mab preparations resulted in a substantial reduction in a number of contaminating proteins including albumin, alpha-2-macroglobulin and transferrin. The Mab bands found near the head of each profile were weak and barely detectable in comparison to bands obtained for more abundant immunoglobulins found in normal human serum (Figure 51b).

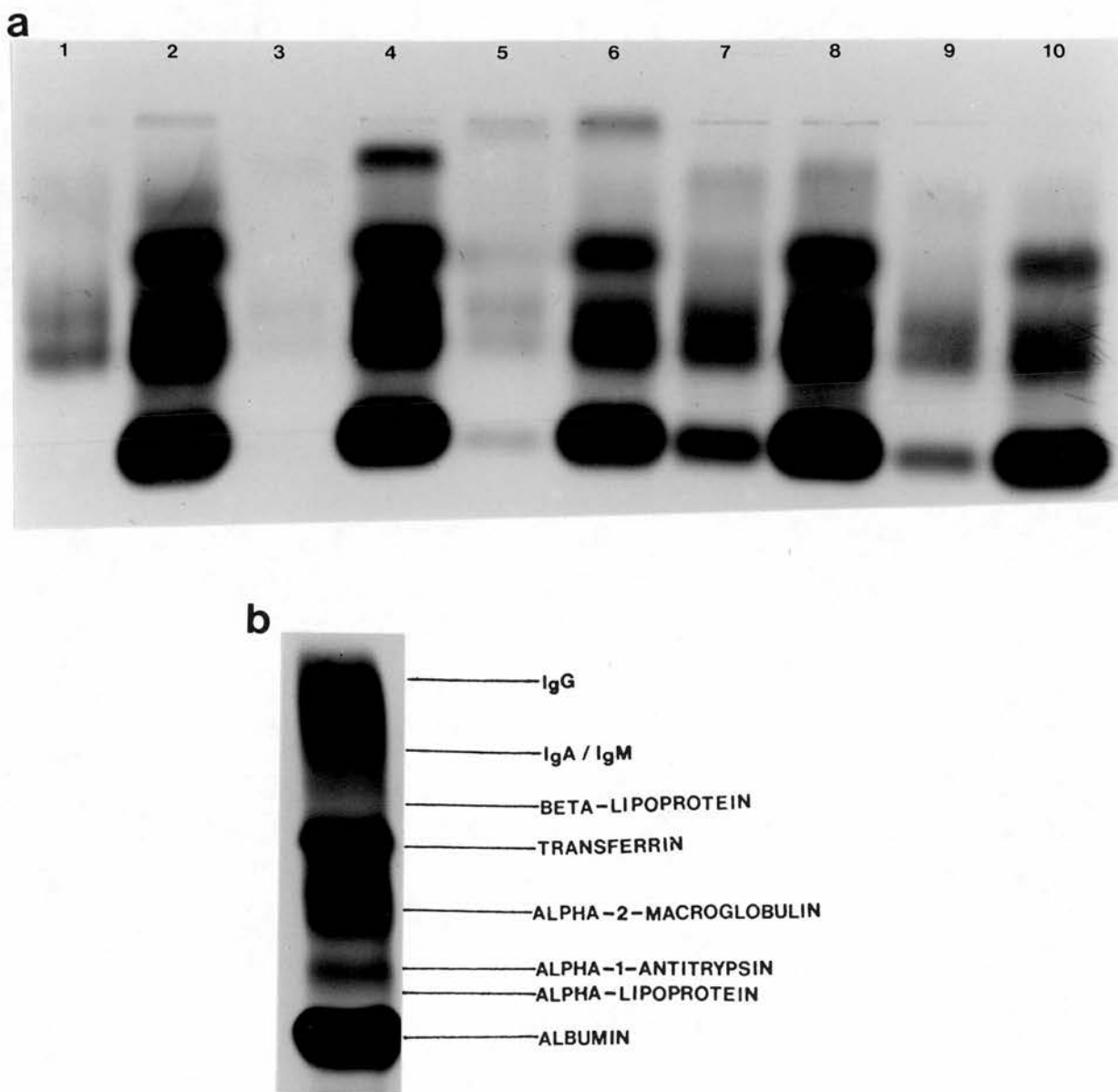


Figure 51. a) Coomassie blue stained profiles of proteins, separated by agarose gel electrophoresis found in concentrated supernatants containing Mab before and after ammonium sulphate precipitation. Track 1, 334.4 (after); Track 2, 334.4 (before); Track 3, 73.5 (after); Track 4, 73.5 (before); Track 5, 61.3.2 (after); Track 6, 61.3.2 (before); Track 7, 360.7 (after); Track 8, 360.7 (before); Track 9, 304.1.4 (after); Track 10, 304.1.4 (before). b) Coomassie blue stained profiles of proteins found in human serum.

CHAPTER 4

LABELLING AND APPLICATIONS OF THE MONOCLONAL ANTIBODIES

4.1 BIOTIN LABELLING OF MONOCLONAL ANTIBODIES

In order to determine the degree of biotinylation of the mouse Mabs which would yield the highest reactivity, various molar ratios of biotin to Mab free amino groups were reacted. These differentially biotinylated Mabs were tested in ELISA against individual cocktails of *P. aeruginosa* R- and S-LPS. In addition, two enzyme streptavidin conjugate systems were investigated:- i) streptavidin-alkaline phosphatase and ii) streptavidin-urease. The results of different biotin: Mab ratios and the two different enzyme conjugates for Mabs 304.1.4 and 360.7 are shown in Figures 52 and 53. All OD readings were expressed after subtraction of the relevant background control.

Conjugation of Mabs 304.1.4 and 360.7 with 1:1, 2:1 or 4:1 biotin: Mab/amino acid ratios appeared to produce usable reagents in each case. The 1:1 ratio gave maximal OD readings for each dilution of Mab. As the dilution of each biotinylated Mab increased, OD readings for wells coated with R-LPS were usually higher than those obtained for wells coated with S-LPS. Since the Mabs recognise unsubstituted core LPS, the increased sensitivity with R-LPS may simply be due to the presence of more available core LPS compared with S-LPS. Comparison of the two streptavidin conjugates showed, for a given dilution of Mab, that OD readings were greater for streptavidin-alkaline phosphatase (Figure 52) than for streptavidin-urease (Figure 53), ie the former is more sensitive.

4.2 BIOTIN-STREPTAVIDIN AMPLIFICATION ELISA SYSTEMS

The sensitivity of different conjugate systems for bio-Mabs used against *P. aeruginosa* S-LPS and R-LPS were compared. The conjugate systems

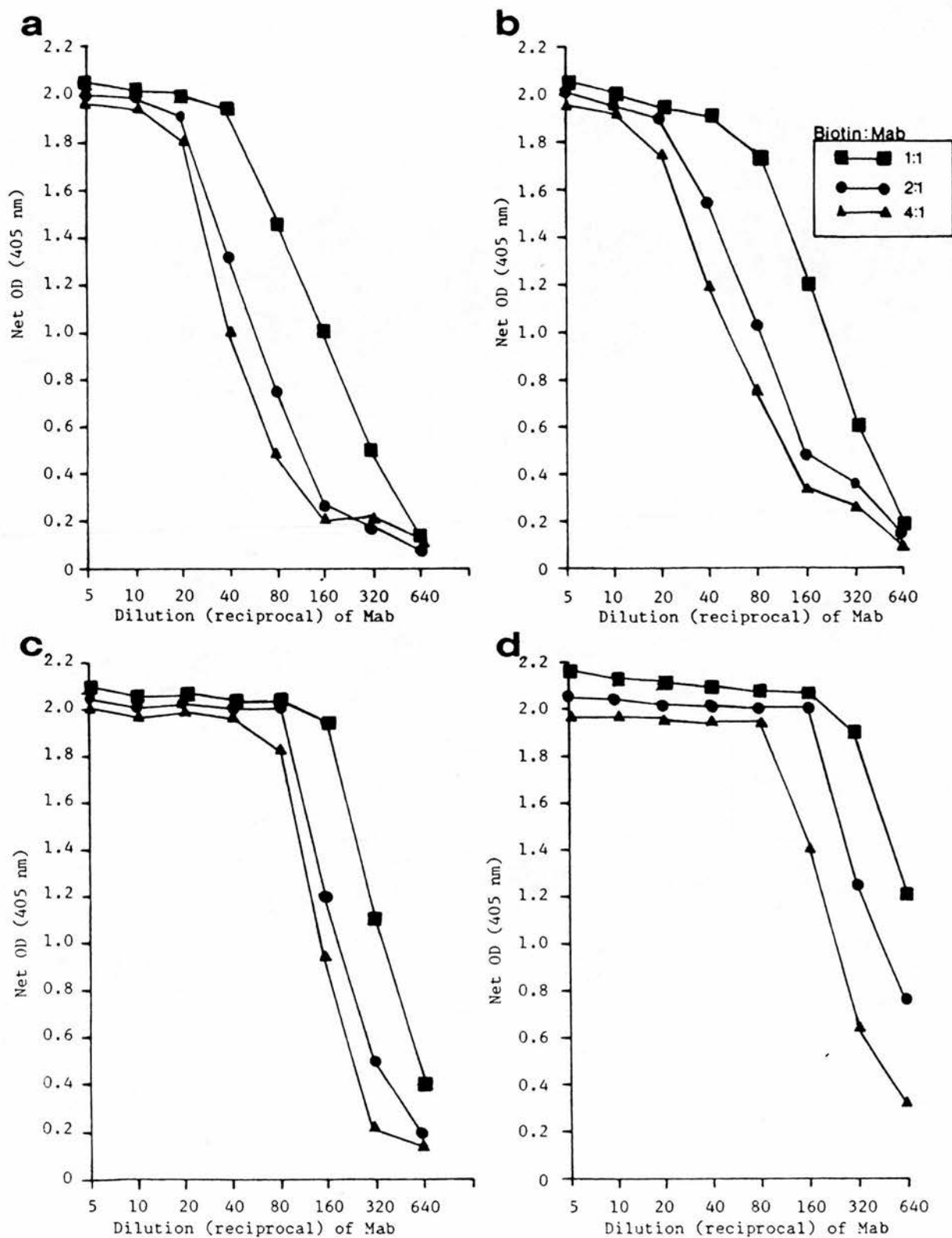


Figure 52. Reactivity in ELISA of Mabs 304.1.4 (a and b) and 360.7 (c and d) coupled with different ratios of biotin. Reactions against a cocktail of smooth (a and c) and rough (b and d) *P. aeruginosa* LPS were measured. Streptavidin-alkaline phosphatase and the corresponding substrate were used to measure the reaction (OD 405 nm).

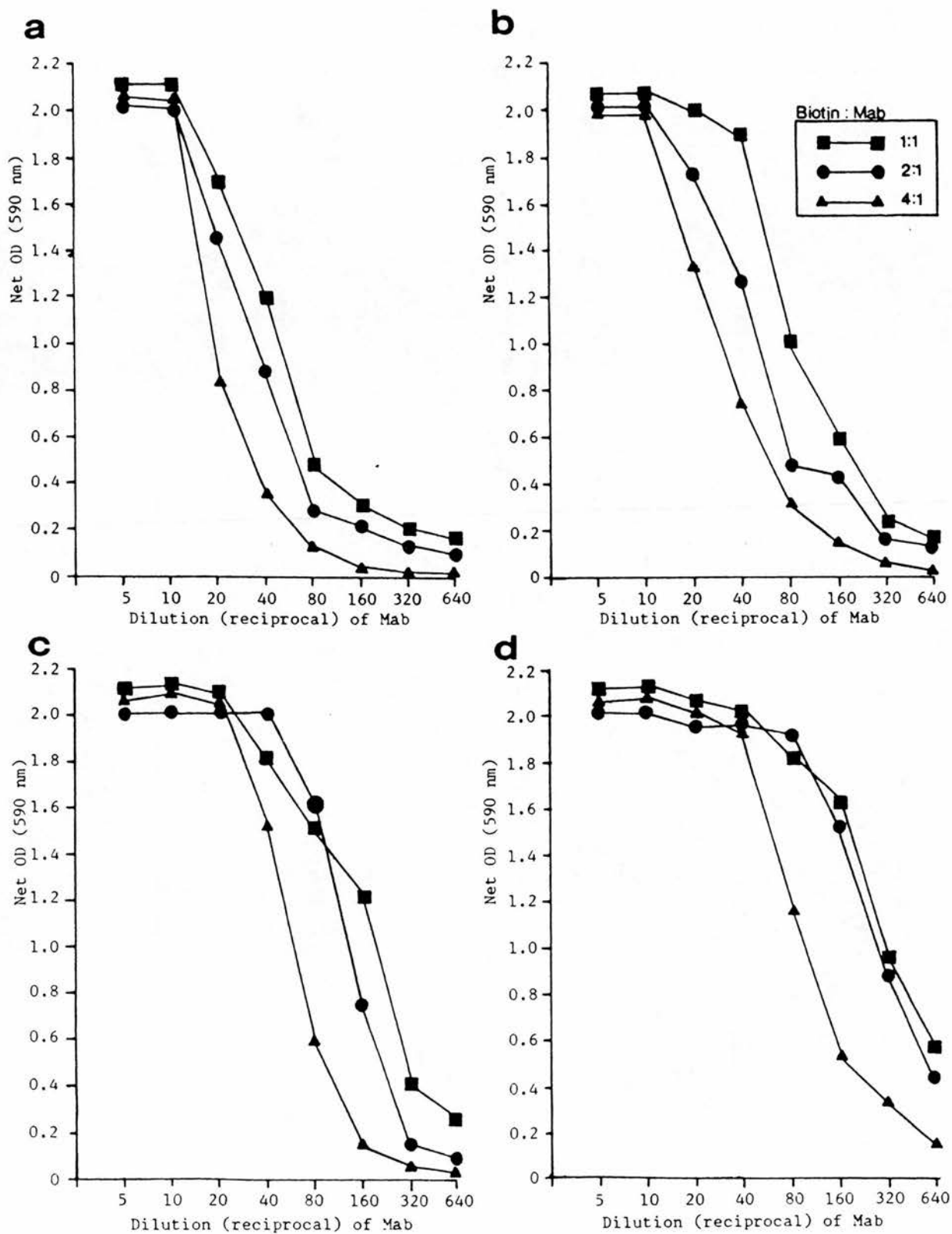


Figure 53. Reactivity in ELISA of Mabs 304.1.4 (a and b) and 360.7 (c and d) coupled with different ratios of biotin. Reactions against a cocktail of smooth (a and c) and rough (b and d) *P. aeruginosa* LPS were measured. Streptavidin-urease and the corresponding substrate were used to measure the reactions (OD 590 nm).

included: i) alkaline phosphatase labelled biotin-streptavidin complex (the amplification system); ii) alkaline phosphatase labelled streptavidin; iii) urease labelled streptavidin.

The results obtained for bio-Mabs (1:1) 304.1.4 and 360.7 with the different conjugate systems are shown in Figure 54. The conjugate system employing alkaline phosphatase labelled biotin-streptavidin complex produced the highest OD values for each respective dilution of bio-Mab (304.1.4 or 360.7) against either *P. aeruginosa* R- and S-LPS. Comparison of the less sensitive non-amplified systems again showed that the alkaline phosphatase labelled streptavidin conjugate was more sensitive than urease labelled streptavidin. Greater sensitivity of the conjugates against *P. aeruginosa* R-LPS was also observed. In addition, Mab 360.7 tended to give higher OD readings than Mab 304.1.4.

The sensitivity of the amplification system (alkaline-phosphatase labelled biotin-streptavidin complex) for *P. aeruginosa* LPS bound to the solid phase of polystyrene microtitre plate wells was investigated (Figure 55). Optimal dilutions of the bio-Mabs (1:1 biotinylation) 304.1.4 and 360.7 were selected from the titration of the Mabs against *P. aeruginosa* S- and R-LPS shown in Figure 54. Bio-Mab 304.1.4 was diluted 1:200 and bio-Mab 360.7 was diluted 1:500. Although these dilutions did not give the maximum possible OD values, they provided strong OD values (OD >1.0) against *P. aeruginosa* R- and S-LPS and helped conserve the amount of bio-Mab used. The bio-Mabs were added separately to wells coated with a doubling dilution series of R-LPS or S-LPS, from 0.08-10.0 ng/ml. A reading was considered significant if the OD value was 3SDs greater than the mean of the background control. The assay system utilizing bio-Mab 304.1.4 and the alkaline phosphatase labelled biotin-streptavidin complex was capable of detecting 0.16 ng/ml R-LPS and 0.08 ng/ml S-LPS. The amplification system incorporating bio-Mab 360.7 was able to detect down to 0.08 ng/ml of R- and S-LPS. In the case of both Mabs, OD

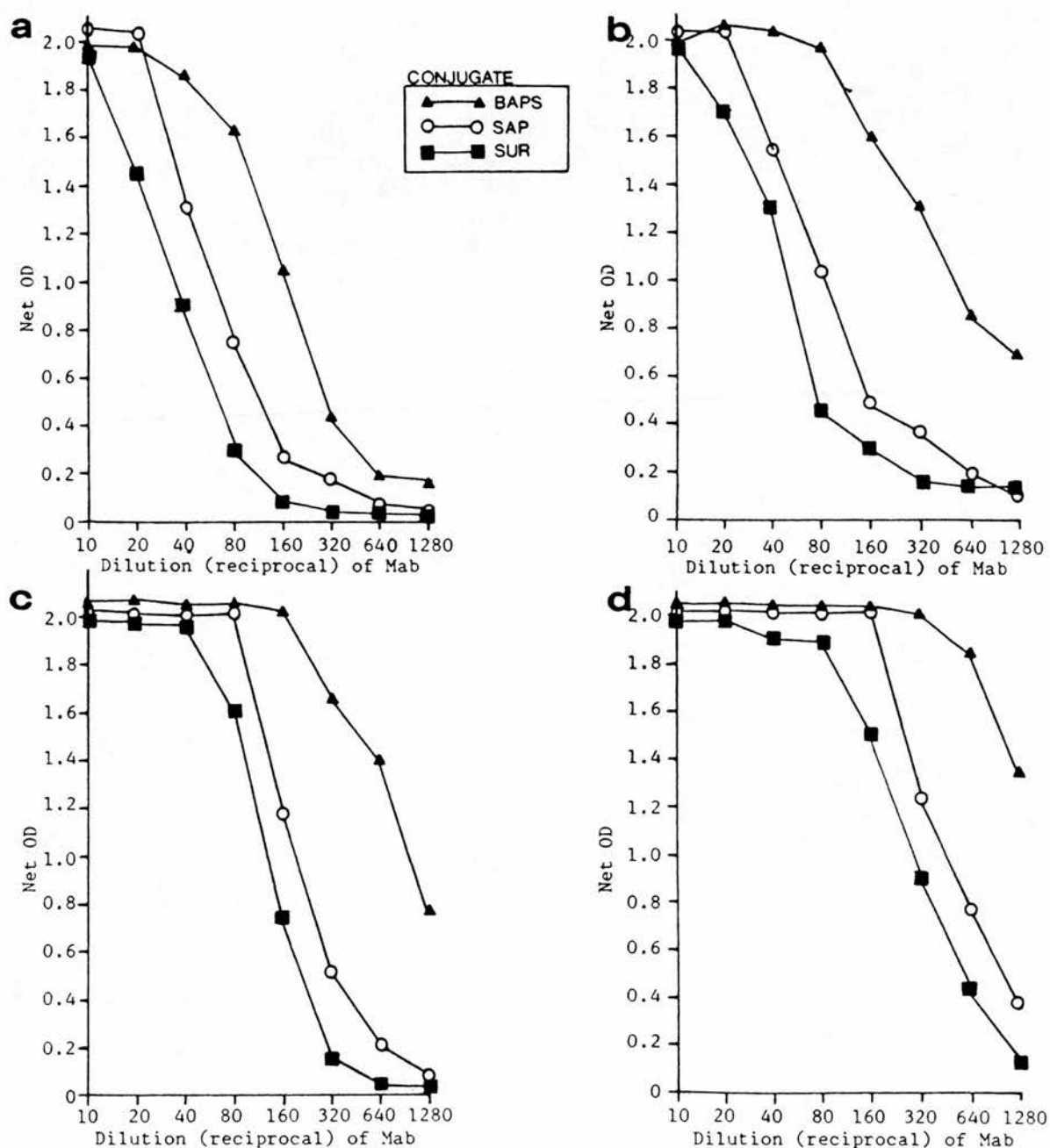


Figure 54. Comparison of the sensitivity of enzyme immunoassays with bio-Mabs 304.1.4 (a and b) and 360.7 (c and d) against smooth (a and c) and rough (b and d) *P. aeruginosa* LPS. The conjugate systems utilized were as follows:- i) alkaline phosphatase labelled biotin streptavidin complex (BAPS); ii) alkaline phosphatase labelled streptavidin (SAP) and iii) urease labelled streptavidin (SUR). The OD values obtained with alkaline phosphatase conjugates were measured at 405 nm; OD values for the urease conjugate were measured at 590 nm.

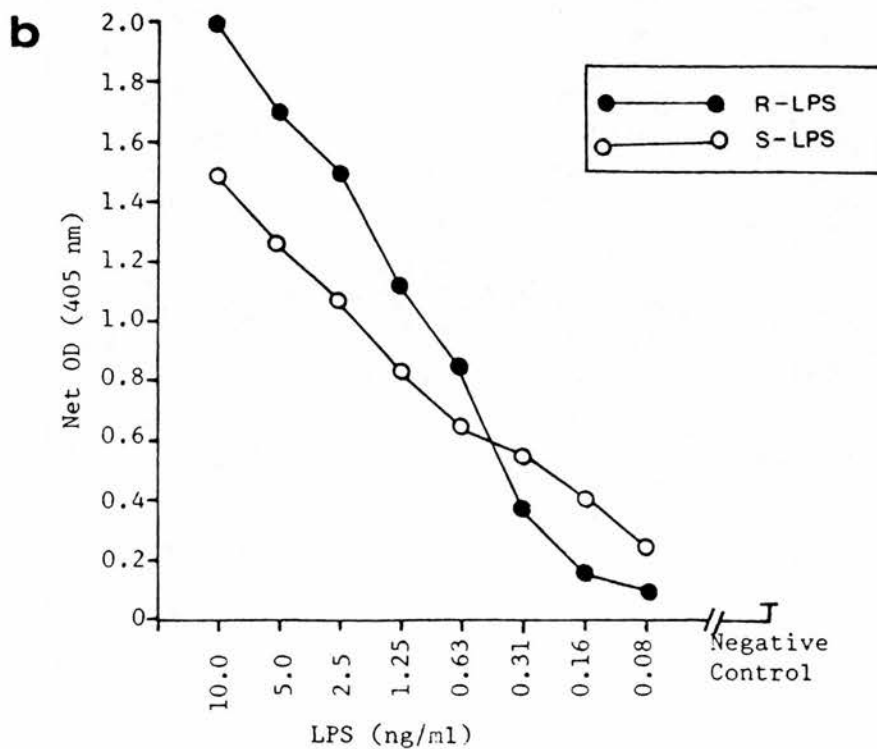
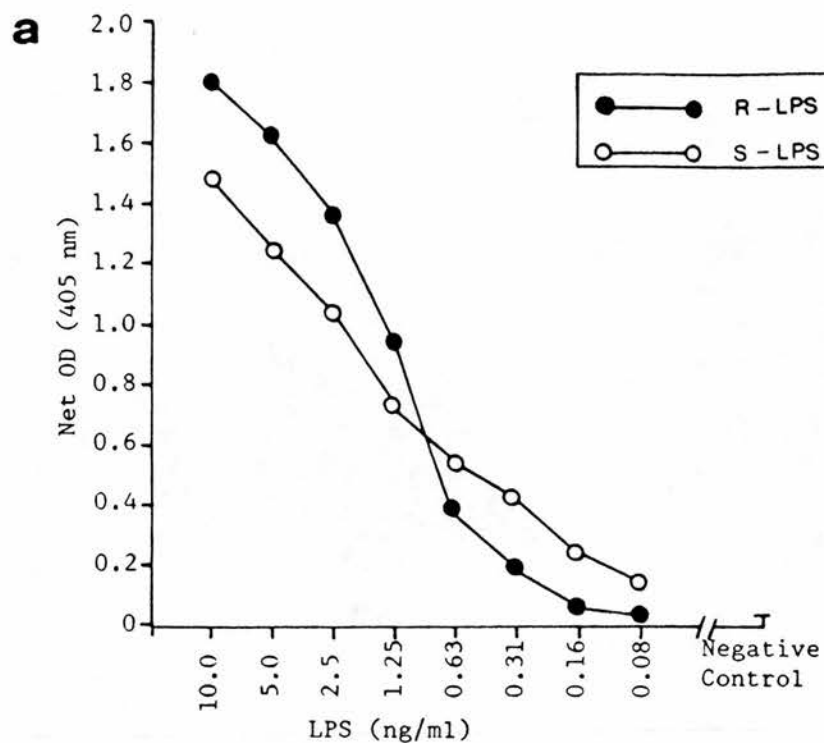


Figure 55. Sensitivity of ELISA for the measurement of *P. aeruginosa* R- and S-LPS bound to the solid phase of microtitre wells. The ELISA system incorporated bio-Mabs a) 304.1.4 (1:200) and b) 360.7 (1:500) and a conjugate system consisting of alkaline phosphatase-labelled biotin-streptavidin complex.

readings were greater for the higher concentrations of R-LPS compared to the same concentrations of S-LPS; however, at the lower concentrations the situation was reversed.

4.3 MONOCLONAL ANTIBODY COMPETITION ASSAYS

The antigen binding sites of the five core LPS specific Mabs were compared by adding various combinations of Mabs (one labelled with biotin and one unlabelled) to wells of microtitre plates coated with core R-LPS. If the labelled antibody and unlabelled antibody bind to separate and discrete sites on the core antigen, the labelled antibody will bind at the same level whether or not the competing antibody is present. However, if the sites of interaction are identical or overlapping, the unlabelled antibody will compete and the amount of labelled antibody bound to the antigen will be lowered.

In order to assess the ability of one Mab to compete with another Mab, a standard curve was established by titrating one Mab against itself, ie the same antibody was used as both the label and the inhibitor (self-inhibition). The capacity of the unlabelled inhibitor to inhibit the binding of other bio-Mabs was also titrated. Doubling dilutions of bio-Mabs were titrated against doubling dilutions of unlabelled inhibitor: the results for bio-Mabs 304.1.4 and 360.7 diluted 1:80, bio-Mabs 61.3.2 and 73.5 diluted 1:10, and bio-Mab 334.4 diluted 1:40 are shown. The results were plotted as shown in Figures 56-58 and the concentration of inhibitor required for 50% inhibition of binding of labelled Mabs was compared. A labelled Mab was considered ^{not} to compete with an unlabelled inhibitor if the dilution of inhibitor, at the 50% inhibition level was at least one dilution less (ie inhibitor more concentrated) than required for self-inhibition. Based on this rationale, Mabs 304.1.4, 360.7 and 73.5 appeared unable to compete one against the other ie Mabs 360.7 and 73.5 could ^{not} compete against 304.1.4 (Figure 56); Mabs 304.1.4 and 73.5 could ^{not} compete against 360.7 (Figure 57) etc. Mab 61.3.2 appeared to follow the binding pattern of Mab 304.1.4 and were considered to share a similar binding site. Bio-Mab 334.4 was

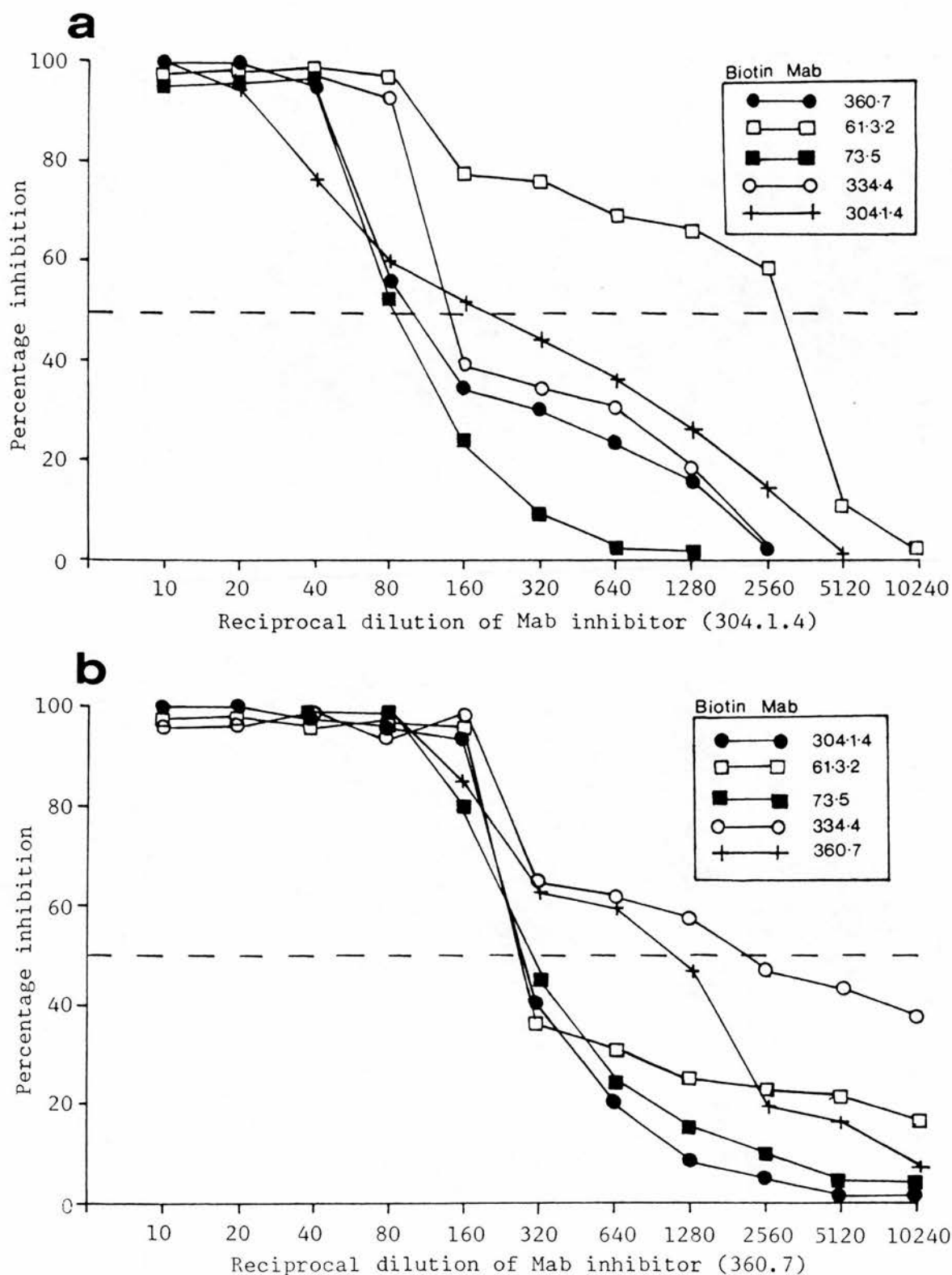


Figure 56. Mab competition assay measured by ELISA (OD 405 nm) with R-LPS from *P. aeruginosa* PAC557 as coating antigen. Doubling dilutions of Mab 304.1.4 (a) and Mab 360.7 (b) inhibitors were run against biotin labelled competing Mabs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie percentage inhibition.

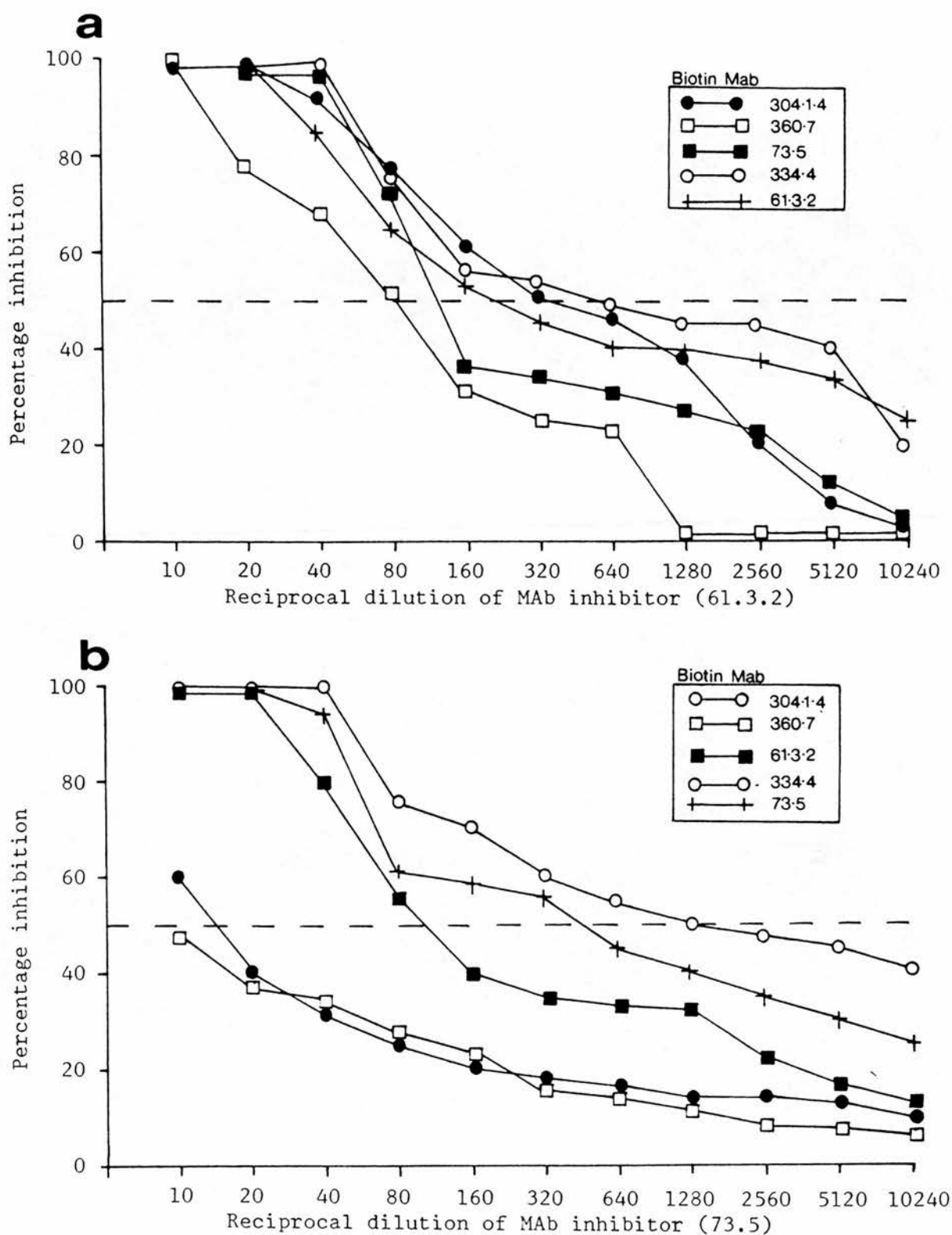


Figure 57. Mab competition assay measured by ELISA (OD 405 nm) with R-LPS from *P. aeruginosa* PAC557 as coating antigen. Doubling dilutions of Mab 61.3.2 (a) and Mab 73.5 (b) inhibitors were run against biotin labelled competing Mabs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie percentage inhibition.

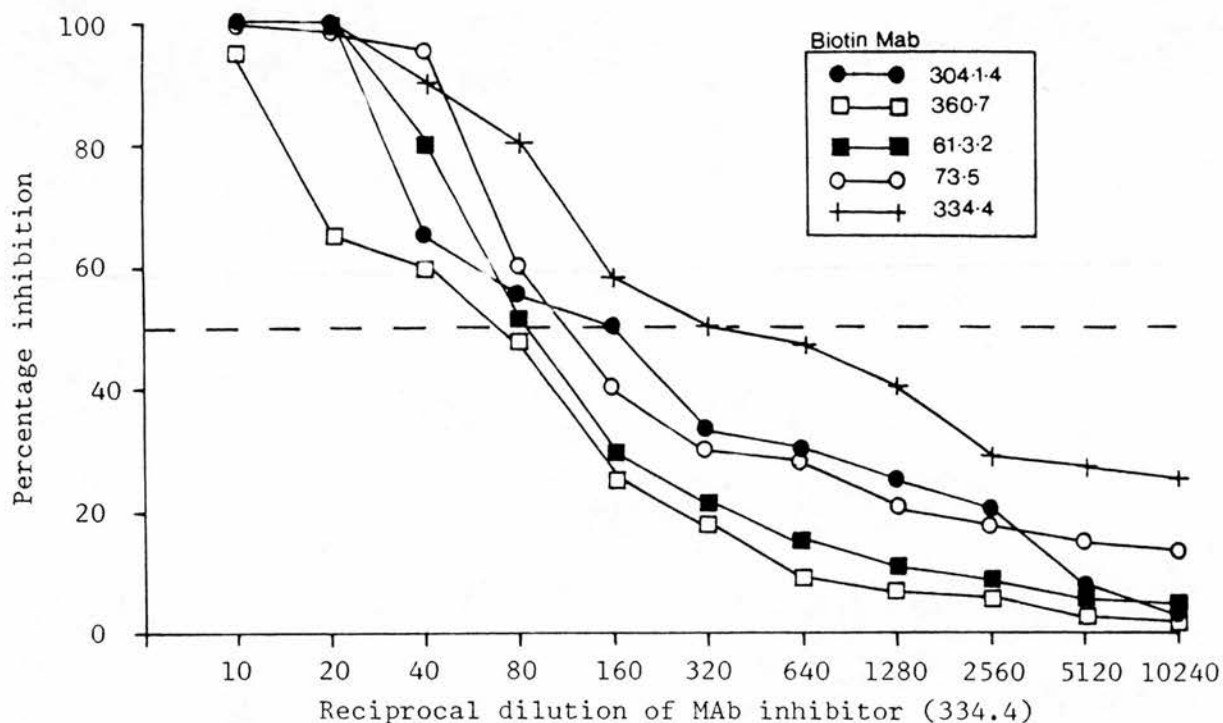


Figure 58. Mab competition assay measured by ELISA (OD 405 nm) with R-LPS from *P. aeruginosa* PAC557 as coating antigen. Doubling dilutions of Mab 334.4 inhibitor were run against the following biotin labelled competing Mabs. The OD values obtained in the presence of inhibitor were calculated as a percentage of OD values obtained without inhibitor, ie percentage inhibition.

able to compete with any of the other unlabelled Mabs, indicating that it may have a similar or overlapping binding site to these Mabs. However, when the other Mabs were biotinylated they appeared^{not} to compete effectively with unlabelled 334.4 inhibitor (Figure 58). This may indicate that Mab 334.4 has a lower affinity for the antigen binding site than the other Mabs.

4.4 CAPTURE ELISA FOR THE DETECTION OF *P. AERUGINOSA* LPS

A capture ELISA was developed for the detection of *P. aeruginosa* LPS in solution. The system developed incorporated the two most cross-reactive, highest affinity Mabs characterised above: 304.1.4 and 360.7 which were also found^{not} to compete against each other. Mab 360.7 was used as the coating/capture antibody since it had the greatest affinity for *P. aeruginosa* LPS. Mab 304.1.4 was used as the conjugated antibody (1:1 biotinylation). The amounts of coating and conjugate antibodies were determined by checkerboard titrations: conjugate antibody was used at 1:200 and coating antibody at 1:25. The capture ELISA incorporated the biotin-streptavidin amplification system investigated earlier. Ten-fold dilutions of *P. aeruginosa* LPS, whole cells and heat-killed cells were tested (Figures 59-61).

Various incubation times (1 h, 2 h, 4 h, overnight) with the added antigens were investigated; an incubation of 2 h gave comparable sensitivity to longer incubations and was used in subsequent experiments. A sample was considered positive for antigen if it yielded an OD value which was 3 SDs greater than the mean of background controls. The sensitivity of the assay was between 0.1- 1.0 ng/ml *P. aeruginosa* LPS eg 0.1 ng/ml for JN61 (S-LPS) and PAC608 (R-LPS) and 1.0 ng/ml for serotypes O:2 and O:11 (S-LPS) (Figure 59). The assay limits of detection with *P. aeruginosa* whole cells was 10^3 - 10^4 cells/ml eg PAC608 (core R-LPS) 10^3 cells/ml, and serotype O:6, 10^4 cells/ml (Figure 60). Similarly, LPS antigen in heat-killed whole cell preparations was detected; sensitivity was 10^3 - 10^4 cells equivalent /ml (Figure 61).

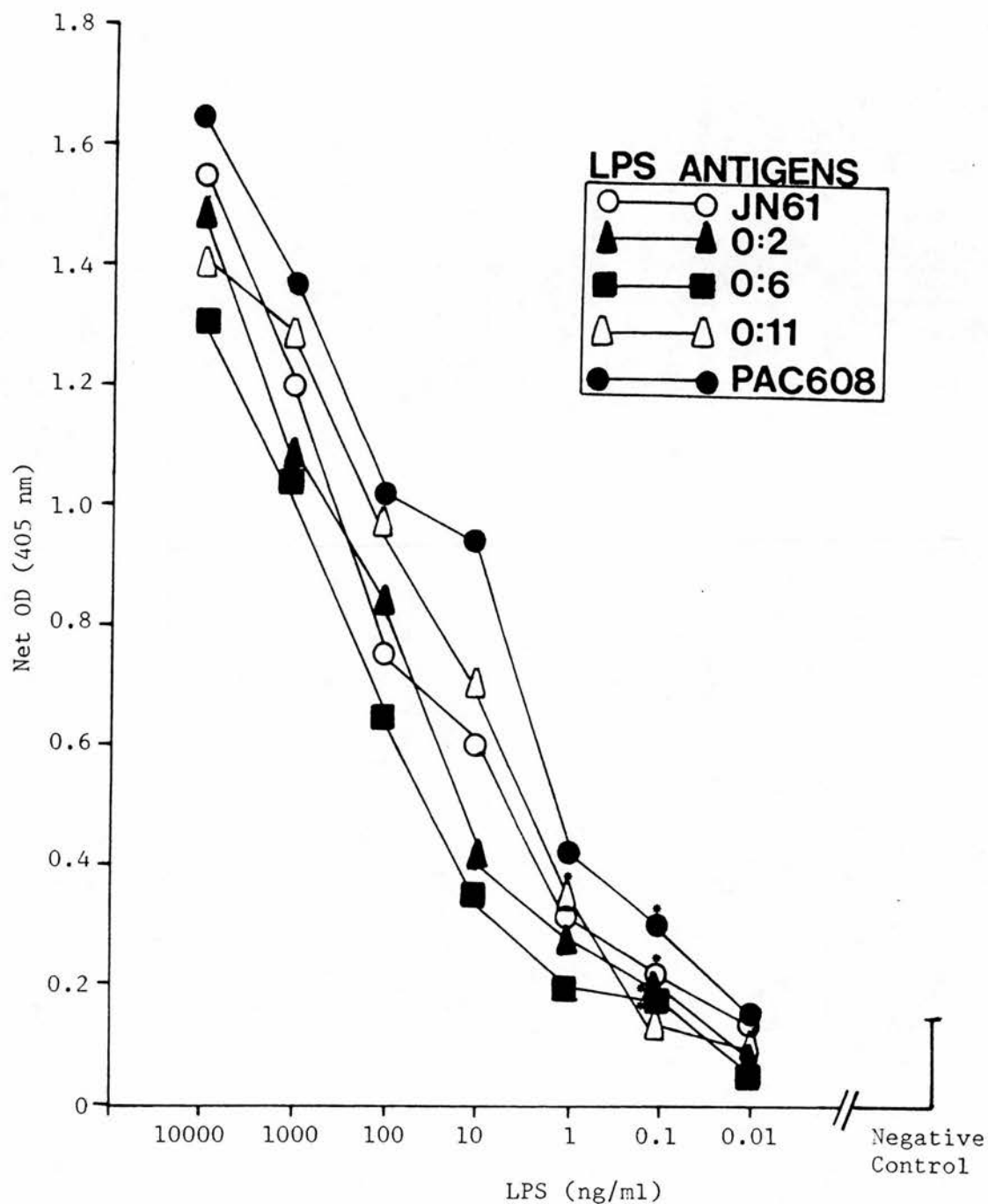


Figure 59. Sensitivity of capture ELISA incorporating a biotin-streptavidin amplification system for the detection of *P. aeruginosa* LPS. The *P. aeruginosa* LPS antigens included: O-antigenic S-LPS from JN61 (non-mucoid, serotype O:6, isolated from patient with CF) and serotype strains O:2, O:6 and O:11, and core R-LPS from PAC608. Concentrations indicated by an asterisk indicate the first value significantly greater than negative control values (>3 SDS of control mean).

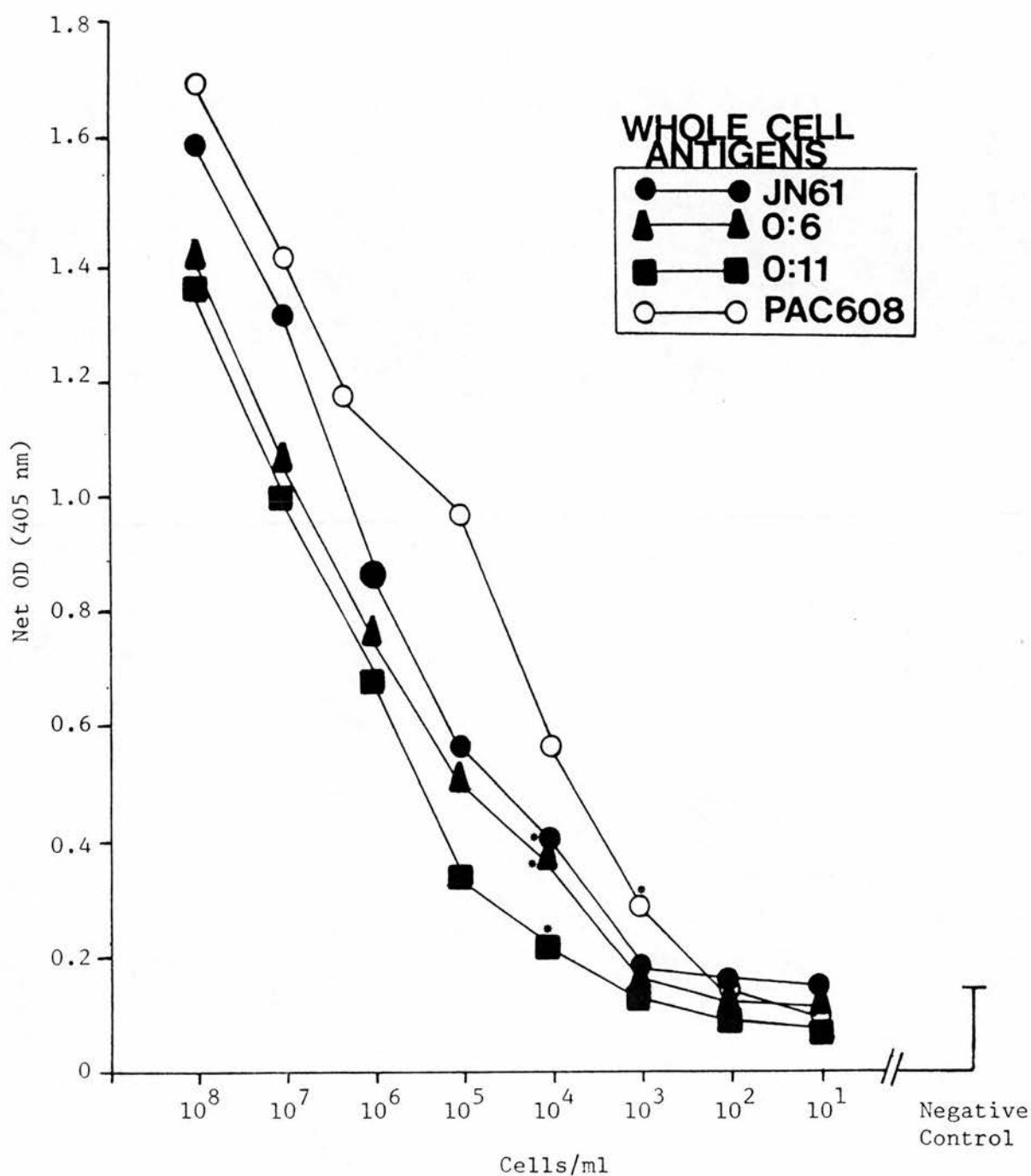


Figure 60. Sensitivity of capture ELISA incorporating a biotin-streptavidin amplification system for the detection of *P. aeruginosa* LPS in solutions containing *P. aeruginosa* whole cells. The *P. aeruginosa* whole cell strains include: JN61 (non-mucoid, serotype O:6 isolated from patient with CF); serotypes O:6 and O:11, and PAC608 (R-LPS strain). Concentrations indicated by an asterisk denote the first value significantly greater than the negative control values (>3 SDs of control mean).

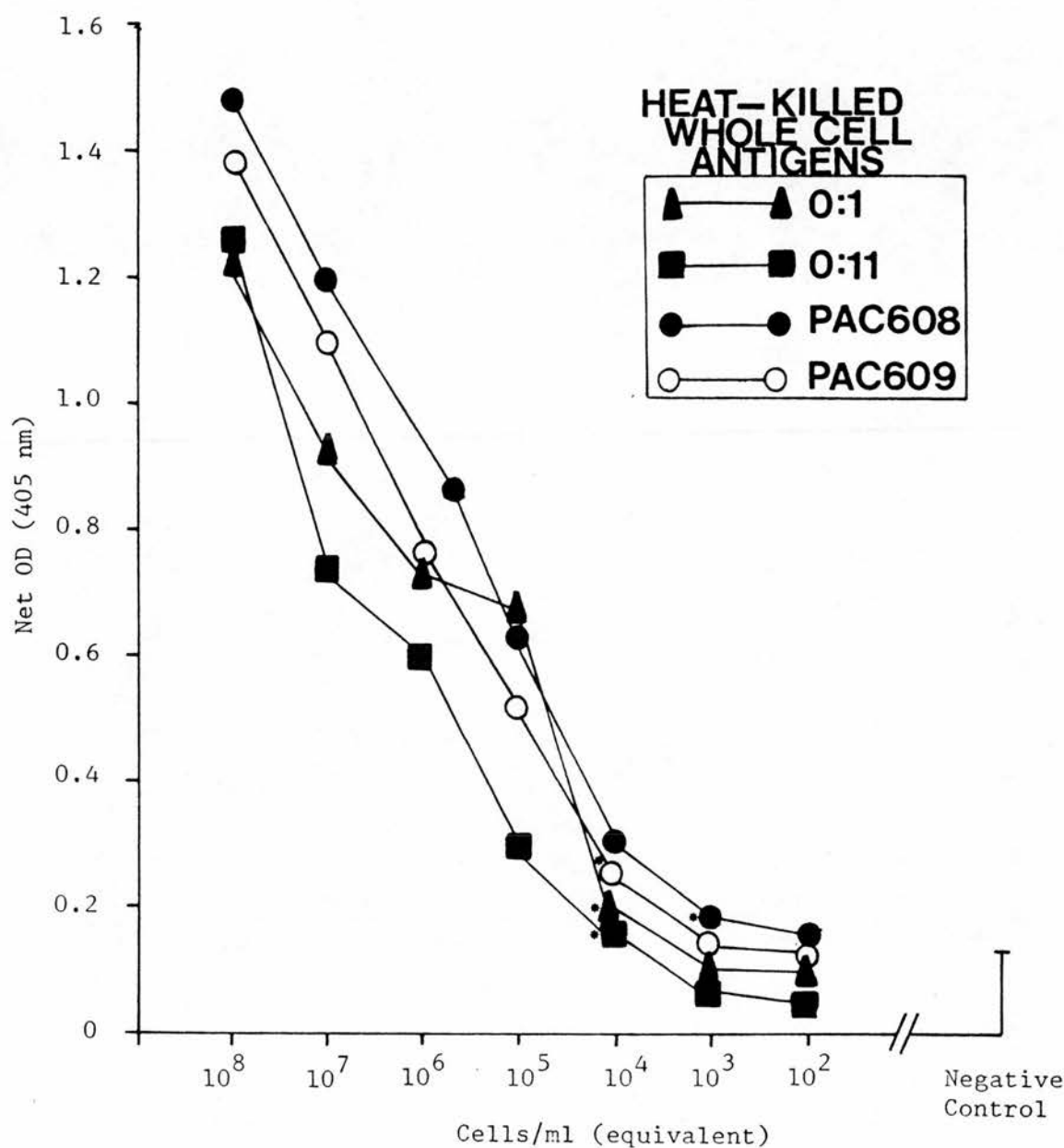


Figure 61. Sensitivity of capture ELISA incorporating a biotin-streptavidin amplification system for the detection of *P. aeruginosa* LPS in solutions containing heat-killed *P. aeruginosa* whole cells. The *P. aeruginosa* heat-killed cell preparations included: O-antigenic S-LPS strains serotypes O:1 and O:11, and core R-LPS strains PAC608 and PAC609. Concentrations indicated by an asterisk indicate the first value significantly greater than negative control values (>3 SDs of control mean).

The capture ELISA system was also applied for the detection of *P. aeruginosa* LPS in saliva and sputum specimens obtained from patients with CF. Specimens were categorised according to the sputum bacteriology of the patient at that particular time: i) colonised with *P. aeruginosa*; ii) Non-*P. aeruginosa* colonised. Boiling of samples prior to use in the assay was found to increase sensitivity of the assay, caused by the deproteinization and subsequent liberation of LPS antigen in samples containing antigen-antibody complexes, and also reduced non-specific background reactions. *P. aeruginosa* LPS was detected in saliva and sputum specimens from both *P. aeruginosa* colonised and non-colonised CF patients (Figure 62). However, false negative values were observed for two sputums and one saliva from CF patients known to be colonised by *P. aeruginosa*. The CF patient classified as non-*P. aeruginosa* colonised (on the basis of sputum bacteriology) but who had the highest sputum OD value (ELISA OD = 1.35) for *P. aeruginosa* LPS in the non-colonised group, subsequently became intermittently colonised by *P. aeruginosa*, as detected by bacteriological culture. Saliva samples from six normal healthy controls were negative for *P. aeruginosa* LPS.

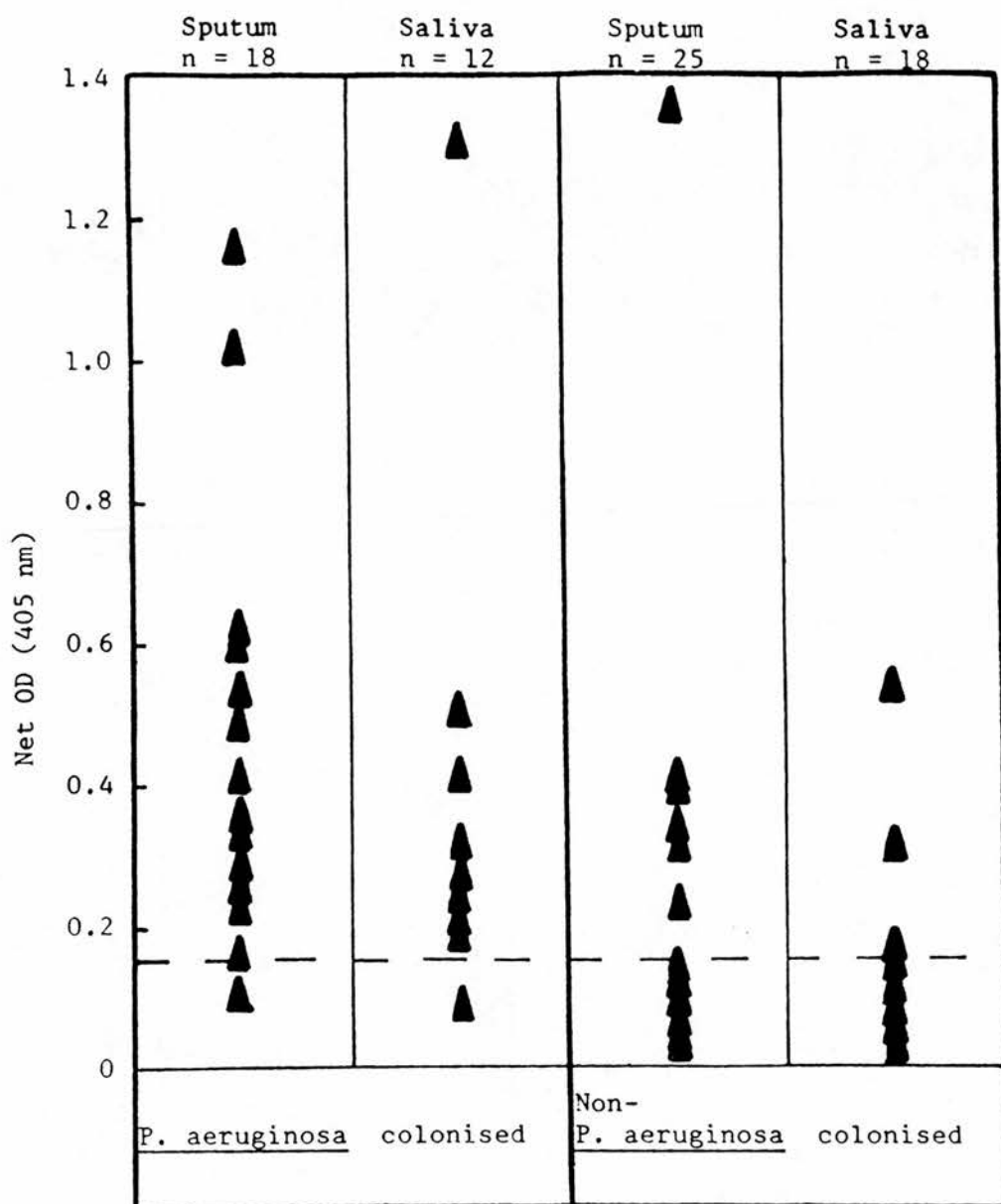


Figure 62. Capture ELISA incorporating a biotin-streptavidin amplification system for the detection of *P. aeruginosa* LPS in sputum and saliva from patients with CF: i) *P. aeruginosa* colonised and ii) non-*P. aeruginosa* colonised. A sample was considered positive for *P. aeruginosa* LPS if it yielded an OD value which was >3 SDs than the mean of background controls (dotted line).

CHAPTER 5

CHEMOTAXIS, MOTILITY AND ADHERENCE OF *P. AERUGINOSA*

The purpose of this work was to investigate the chemotactic and adherence properties of non-mucoid *P. aeruginosa* strains isolated from patients with CF, and to analyse the possible role these factors may play in the initial stages of pulmonary colonisation by the organism.

5.1 *P. AERUGINOSA* STRAINS USED IN CHEMOTAXIS EXPERIMENTS

The chemotactic response of ten individual strains of *P. aeruginosa*, towards various mucin preparations and the major amino acid and sugar components of mucin were investigated. The strains used in the studies included:

- a) The standard genetic strain *P. aeruginosa* PAO1.
- b) *P. aeruginosa* strains J1385 and J1375, which were isolated during an investigation into the primary respiratory colonisation in two CF patients who had bathed in a hydrotherapy pool contaminated with multiple strains of *P. aeruginosa*. J1385 is the colonising strain which was isolated from both the CF patients and from the pool water. J1375 represents one of the four other environmental isolates taken from the water. The clonal relation of these isolates was determined by pyocin typing and confirmed by DNA probe analysis by Dr M.L. Vasil and Dr J.W. Ogle (Health Sciences Center, Denver, Colo. USA).
- c) *P. aeruginosa* strains JN8, JN47, JN61 and JN62 were isolated from the sputa of four CF patients with no previous history of pulmonary colonisation by *P. aeruginosa*.
- d) *P. aeruginosa* strains WR-5, a non-chemotactic mutant; M2Fla⁻, a non-flagellate mutant and M2Rev, the motile revertant of M2Fla⁻.

In all chemotaxis experiments a meaningful chemotactic response was defined as a relative response >2.0.

5.2 CHEMOTACTIC RESPONSES OF *P. AERUGINOSA* TO PORCINE MUCIN

In the initial studies the chemotactic response of *P. aeruginosa* towards a commercial preparation of porcine gastric mucin was investigated (Table 4). All strains except non-motile M2Fla⁻ and non-chemotactic WR-5 exhibited a meaningful chemotactic response towards porcine gastric mucin at some point over the 60 minute assay period. The three highest relative responses were obtained by *P. aeruginosa* strains J1385 (12.7), PAO1 (10.6) and JN47 (10).

5.3 CHEMOTACTIC RESPONSES OF *P. AERUGINOSA* TO PURIFIED CF MUCINS

Chemotaxis of *P. aeruginosa* to purified mucins derived from the tracheobronchial secretions of a patient with CF were investigated (Tables 5-9). The chemotactic responses of *P. aeruginosa* strains towards purified CF (gel fraction) mucin are shown in Table 5. All strains except negative controls demonstrated a meaningful chemotactic response towards CF mucin. The three highest peak responses were obtained by *P. aeruginosa* strains J1385 (17.2), PAO1 and J1375 (6.7).

In subsequent experiments the chemotactic response of *P. aeruginosa* towards individual fractions of CF gel mucin was investigated to determine if any of the fractions were particularly chemoattractive for *P. aeruginosa* (Tables 6-9). A meaningful response towards each of CF mucin fractions (A-D) was obtained for most of the *P. aeruginosa* strains, excluding negative controls. All strains showed a meaningful response towards gel fraction B of CF mucin (Table 7), which appeared to be the most effective chemoattractant of the four fractions studied. Certain strains showed a meaningful response to only some of the mucin fractions, eg JN47 showed meaningful responses to fractions B and D

Table 4. Chemotactic responses of P. aeruginosa strains towards porcine-gastric mucin (100 µg/ml) over a period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	10.6	4.5	1.5	3.2
J1385	2.4	12.7	4.9	1.8
J1375	6.1	3.7	0.9	1.1
JN8	4.0	3.0	4.6	1.9
JN47	0.8	10.0	0.6	0.2
JN61	3.7	2.6	1.7	0.5
JN62	1.6	3.0	2.6	5.8
M2Rev	1.8	4.6	0.3	1.2
M2Fla ⁻	0.9	0.3	1.0	0.2
WR-5	0.2	1.5	0.6	0.9

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to control capillaries containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 5. Chemotactic responses of P. aeruginosa strains towards CF tracheobronchial gel mucin (100 µg/ml) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	2.7	11.6	1.1	0.8
J1385	17.2	0.4	3.8	2.3
J1375	0.8	1.8	6.7	2.9
JN8	5.1	1.7	0.9	0.8
JN47	3.5	0.7	0.5	1.0
JN61	0.4	4.3	3.4	1.5
JN62	0.5	3.3	1.3	1.1
M2Rev	1.3	2.8	1.5	0.9
M2Fla ⁻	0.5	1.8	0.8	0.7
WR-5	0.8	1.3	1.0	0.8

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 6. Chemotactic responses of P. aeruginosa strains towards CF tracheobronchial mucin gel fraction A (100 µg/ml) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	1.8	1.2	4.0	1.9
J1385	3.4	0.6	0.5	0.9
J1875	4.8	1.2	2.5	2.1
JN8	0.5	2.0	0.9	0.8
JN47	0.9	0.3	0.4	0.8
JN61	1.2	1.8	2.2	2.0
JN62	4.0	0.3	2.9	2.0
M2Rev	1.1	2.5	2.2	0.9
M2Fla ⁻	1.5	1.0	0.7	0.8
WR-5	1.7	1.1	1.5	0.9

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 7. Chemotactic responses of P. aeruginosa strains towards CF tracheobronchial mucin gel fraction B (100 µg/ml) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	4.0	2.1	1.1	1.0
J1385	12.5	1.3	1.4	1.2
J1375	1.2	4.8	3.0	1.9
JN8	10.9	6.2	5.6	3.3
JN47	1.6	5.6	1.1	1.3
JN61	1.8	3.4	4.2	2.2
JN62	5.0	1.4	0.5	0.9
M2Rev	4.4	1.1	1.3	1.5
M2Fla ⁻	1.5	0.2	1.3	1.4
WR-5	1.2	1.3	1.0	1.1

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 8. Chemotactic responses of P. aeruginosa strains towards CF tracheobronchial mucin gel fraction C (100 µg/ml) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	5.6	1.3	2.2	2.1
J1385	0.3	1.4	1.3	1.6
J1375	5.0	1.0	3.2	1.4
JN8	6.0	2.3	1.1	1.4
JN47	0.8	0.7	0.3	0.9
JN61	0.5	0.8	1.5	1.1
JN62	1.1	0.3	4.7	2.6
M2Rev	1.5	3.1	2.2	1.7
M2Fla ⁻	1.4	1.5	1.2	1.0
WR-5	1.8	1.9	1.4	1.6

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 9. Chemotactic responses of P. aeruginosa strains towards CF tracheobronchial mucin gel fraction D (100 µg/ml) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	6.5	3.2	4.6	3.0
J1385	0.6	7.6	4.0	2.1
J1375	1.6	1.5	4.1	2.9
JN8	1.3	3.5	1.6	1.7
JN47	3.2	0.6	0.6	0.9
JN61	0.2	0.6	0.6	0.7
JN62	1.1	1.0	1.7	1.5
M2Rev	1.4	4.0	2.5	2.1
M2Fla ⁻	1.2	1.4	1.3	0.7
WR-5	2.1	2.0	2.2	1.2

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

whilst JN61 showed responses only to fractions A and C. The strains which obtained the highest response for each CF mucin fraction were: fraction A - J1375 (4.8); fractions B and D - J1385 (12.5 and 7.6) and fraction C - JN8 (6.0).

5.4 CHEMOTACTIC RESPONSES OF *P. AERUGINOSA* TO PURIFIED HUMAN, NON-CF MUCINS

Chemotaxis of *P. aeruginosa* to other human mucins, derived from non- CF sources were also investigated (Tables 10 & 11). A meaningful, strain dependent chemotactic response towards chronic bronchitic mucin was observed (Table 10). The three highest responses were obtained by JN8 (8.2), J1385 (5.6) and JN61 (4.0). Similarly, meaningful chemotactic responses were obtained for all strains, except negative controls, towards salivary mucins obtained from a normal healthy individual (Table 11). The three highest responses were obtained by J1375 (7.6), PAO1 (7.4) and JN62 (6.0).

5.5 CHEMOTACTIC RESPONSES OF *P. AERUGINOSA* TO MUCIN FRAGMENTS

The chemotactic response of individual strains of *P. aeruginosa* to CF mucin glycopeptides (gel) was investigated to establish the presence, or otherwise, of an enhanced response to low molecular weight mucins compared to those of high molecular weight. (The CF gel glycopeptides have a M_r of between 300-500,000, compared to $10-12 \times 10^6$ for CF gel mucins). In addition, salivary mucins and CF gel mucin (fraction B) were pretreated with pronase for 24 h prior to use in chemotaxis assays in order to determine whether or not the smaller fragments enhanced the chemotactic response of *P. aeruginosa*. (The naked peptide regions of human tracheobronchial mucins are extensively degraded by pronase (Vishwanath & Ramphal, 1985)).

Meaningful chemotaxis towards CF gel glycopeptides was observed for all strains examined except non-motile and non-chemotactic controls (Table 12). The three peak responses were obtained with J1385 (7.4), PAO1 (6.5) and

Table 10. Chemotactic responses of P. aeruginosa strains towards chronic bronchitic mucin (100 µg/ml) over a period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	2.4	0.9	1.8	1.0
J1385	5.6	1.1	0.4	0.8
J1375	2.1	0.5	0.9	0.9
JN8	8.2	7.1	0.5	1.1
JN47	2.8	1.2	0.4	0.9
JN61	0.8	2.6	4.0	1.8
JN62	2.3	3.0	0.6	1.2
M2Rev	2.2	2.4	1.8	1.1
M2Fla ⁻	1.4	1.5	0.7	0.8
WR-5	1.0	1.3	1.8	1.3

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 11. Chemotactic responses of *P. aeruginosa* strains towards salivary mucins (large) (100 µg/ml) over a period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	7.4	4.8	4.8	3.0
J1385	0.6	5.5	2.9	2.5
J1375	7.6	1.0	1.9	1.5
JN8	4.3	4.9	1.6	1.2
JN47	6.3	1.0	0.6	1.1
JN61	1.2	3.0	0.3	0.7
JN62	6.0	3.3	1.3	1.2
M2Rev	2.9	2.5	1.0	0.9
M2Fla ⁻	2.2	0.7	0.8	0.6
WR-5	0.9	1.2	1.2	1.1

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

J1375/JN8 (5.0) which mirror the responses with CF gel mucin. An enhanced chemotactic response with glycopeptides compared to CF mucin (gel) was not observed.

Similarly, treatment of salivary or CF (gel fraction B) mucins with pronase, prior to use in chemotaxis assays, did not appear to significantly affect the chemotactic response of the *P. aeruginosa* strains compared to the untreated mucin, ie responses before and after pronase treatment of mucins were similar, indicating that degradation of mucins into smaller fragments did not enhance or decrease the chemotactic response (Tables 13 and 14).

5.6 SUMMARY OF THE CHEMOTACTIC RESPONSES OF *P. AERUGINOSA* TO MUCINS

Table 15 shows the peak chemotactic response for each of the strains against each of the mucins studied. All *P. aeruginosa* strains including standard strains PAO1 and M2Rev, and the non-mucoid, colonising strains isolated from patients with CF demonstrated chemotactic tendencies towards mucin. Chemotaxis was not specifically towards CF mucin or human mucins in general, since strains also demonstrated meaningful chemotaxis towards porcine-gastric mucin. *P. aeruginosa* J1385, the colonising strain, showed a greater chemotactic response compared to J1375 (environmental strain) to six out of the nine mucin preparations.

5.7 CHEMOTAXIS TO MUCIN-ASSOCIATED AMINO ACIDS AND SUGARS

The ability of the major amino acid and sugar components of mucin to act as chemoattractants was investigated. The chemotactic responses of *P. aeruginosa* towards the eight major amino acids present in mucin, namely alanine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and threonine, at concentrations 10^{-1} - 10^{-5} M were measured. For most of the amino acids examined, considerable differences in the relative chemotactic response of

Table 12. Chemotactic responses of P. aeruginosa strains towards CF mucin gel glycopeptides (100 µg/ml) over a period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	0.9	6.5	4.1	2.2
J1385	1.0	7.4	0.3	0.9
J1875	5.6	1.5	2.6	1.8
JN8	5.6	0.8	1.0	1.3
JN47	3.7	0.9	1.0	1.1
JN61	2.9	1.0	1.6	1.0
JN62	4.3	5.0	2.2	2.0
M2Rev	4.1	1.1	0.4	0.7
M2Fla ⁻	1.0	0.6	0.8	0.9
WR-5	0.9	0.8	0.7	0.8

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. M2Fla⁻ = non-flagellate control.

c. WR-5 = non-chemotactic control.

Table 13. Chemotactic responses of *P. aeruginosa* strains towards salivary mucins (large) (100 µg/ml) pretreated with pronase (100 µg/ml for 24 h at 37°C) over a period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	6.7 (7.4)	4.4 (4.8)	4.1 (4.8)	2.5 (3.0)
J1385	0.4 (0.6)	3.7 (5.5)	0.8 (2.9)	2.1 (2.5)
J1375	5.0 (7.6)	0.7 (1.0)	2.1 (1.9)	1.9 (1.5)
JN8	3.4 (4.3)	5.1 (4.9)	1.6 (1.6)	1.5 (1.2)
JN47	3.5 (6.3)	0.3 (1.0)	0.2 (0.6)	0.9 (1.1)
JN61	1.6 (1.2)	3.2 (3.0)	0.7 (0.3)	0.9 (0.7)
JN62	8.0 (6.0)	4.0 (3.3)	0.9 (1.3)	1.0 (1.2)

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. Figure in bold represents the chemotactic response obtained after treatment of mucin with pronase.

c. Figure in brackets represents the chemotactic response obtained in the absence of pronase treatment.

Table 14. Chemotactic responses of *P. aeruginosa* strains towards CF tracheobronchial mucin gel fraction B (100 µg/ml) pre-treated with pronase (100 µg/ml for 24 h at 37°C) over an assay period of 60 min.

STRAIN	TIME (Min)			
	15	30	45	60
PAO1	3.9 (4.0)	1.4 (2.1)	2.0 (1.1)	1.2 (1.0)
J1385	8.6 (12.5)	1.4 (1.3)	2.7 (1.4)	1.2 (1.2)
J1375	2.0 (1.2)	3.5 (4.8)	2.4 (3.0)	1.6 (1.9)
JN8	10.4 (10.9)	3.7 (6.2)	3.5 (5.6)	2.5 (3.3)
JN47	1.0 (1.6)	5.2 (5.6)	0.9 (1.1)	1.7 (1.3)
JN61	1.5 (1.8)	2.5 (3.4)	5.0 (4.2)	2.7 (2.2)
JN62	5.5 (5.0)	3.8 (1.4)	1.4 (0.5)	1.6 (0.9)
M2Rev	10.9 (4.4)	4.3 (1.1)	1.2 (1.3)	1.8 (1.5)
M2Fla ⁻	1.8 (1.5)	1.4 (0.2)	1.5 (1.3)	1.0 (1.4)
WR-5	0.8 (1.2)	1.1 (1.3)	1.6 (1.0)	1.5 (1.1)

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. The mean from duplicate experiments is shown. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. Figure in bold represents the chemotactic response obtained after treatment of mucin with pronase.

c. Figure in brackets represents the chemotactic response obtained in the absence of pronase treatment.

d. M2Fla⁻ = non-flagellate control.

e. WR-5 = non-chemotactic control.

Table 15. A summary - the peak chemotactic responses of P. aeruginosa strains towards mucin preparations (100 µg/ml)

STRAIN	Porcine-gastric mucin	Chemotactic Response ^a towards:							
		CF (gel) mucin	CF (gel) mucin Fraction A	CF (gel) mucin Fraction B	CF (gel) mucin Fraction C	CF (gel) mucin Fraction D	Chronic bronchitic mucin	Normal Salivary mucin	Gel glycopeptide
PA01	10.6 ^b	11.6	4.0	4.0	5.6	6.5	2.4	7.4	6.5
J1385	12.7	17.2	3.4	12.5	3.2	7.6	5.6	5.5	7.4
J1375	6.1	6.7	4.8	4.8	5.0	4.1	2.1	7.6	5.6
JN8	4.6	5.1	2.0	10.9	6.0	3.5	8.2	4.9	5.6
JN47	10.0	3.5	NC	5.6	NC ^c	3.2	2.8	6.3	3.7
JN61	3.7	4.3	2.2	4.2	NC	NC	4.0	3.0	2.9
JN62	5.8	3.3	4.0	5.0	4.7	NC	3.0	6.0	5.0
M2Rev	4.6	2.8	2.5	4.4	3.1	4.0	2.4	2.9	4.1
M2Fla ^{-d}	NC	NC	NC	NC	NC	NC	NC	2.2	NC
WR-5 ^e	NC	NC	NC	NC	NC	2.2	NC	NC	NC

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing mucin to accumulation of bacteria in a control capillary containing no chemoattractant. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. The highest relative chemotactic response for each mucin obtained over a 60 min assay period is shown. The response value shown represents the mean from duplicate assays.

c. NC = no chemotaxis (ie <2.0).

d. M2Fla⁻ = non-flagellate control.

e. WR-5 = non-chemotactic control.

each of the strains examined were observed (Tables 16a & b). Chemoattraction to serine, alanine, glycine, proline and threonine was particularly evident; with most of the amino acids the peak chemotactic response occurred at a concentration of 10^{-3} M.

Chemotaxis assays were also performed to assess the potential of the major sugars of mucin, namely L-fucose, D-galactose, GalNAc, GlcNAc and NANA to act as chemoattractants for *P. aeruginosa*. The relative chemotactic responses for the strains (Table 17) reflected the results of other chemotaxis assays, emphasizing a strain-dependent response to L-fucose, D-galactose, GalNAc and GlcNAc. No strain showed a chemotactic response to NANA (data not shown). Comparison of the chemotactic responses measured for mucin associated sugars and amino acids, indicated that the former were less effective chemoattractants than the latter.

5.8 COMPARISON OF THE CHEMOTAXIS OF NON-MUCOID AND MUCOID *P. AERUGINOSA* STRAINS

The chemotactic responses of non-mucoid and mucoid *P. aeruginosa* isolates, JN9 and JN10 respectively, were compared to assess the possible effect of mucoidy on chemotaxis. JN9 and JN10 were isolated from the same CF patient and their clonal relationship confirmed by pyocin typing. The chemotactic responses towards mucin associated amino acids and sugars were measured (Figure 63). Both strains demonstrated comparably meaningful (>2.0) responses towards proline and threonine. However, only non-mucoid strain JN9 demonstrated a meaningful response towards serine and the sugars fucose and GlcNAc. The response of JN9 towards serine was approximately five-fold greater than that obtained for mucoid strain JN10.

Table 16a. Chemotactic responses of P. aeruginosa strains towards mucin-associated amino acids at concentrations of 10^{-1} - 10^{-5} M

STRAIN	Alanine					Aspartate				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	2.4	2.6	2.3	NC	NC	NC	NC	NC	NC
J1385	2.4	3.9	7.2	5.3	2.7	NC	4.1	5.0	2.7	NC
J1375	NC	3.2	4.3	4.0	3.5	NC	2.4	2.5	2.3	NC
JN8	4.0	3.1	5.7	12.4	NC	NC	NC	NC	NC	NC
JN47	2.4	4.8	5.0	2.7	2.1	NC	NC	NC	2.0	NC
JN61	7.7	7.6	8.2	5.5	3.0	2.4	2.7	3.1	NC	NC
JN62	2.5	2.1	3.3	5.5	3.1	NC	NC	NC	2.1	NC
M2Rev	2.1	2.5	3.3	3.0	2.7	NC	NC	NC	NC	NC
M2F1a ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

STRAIN	Cysteine					Glutamate				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	NC	NC	NC	NC	NC	NC	2.3	2.2	2.2
J1385	NC	NC	2.2	NC	NC	NC	4.0	6.5	2.9	2.5
J1375	NC	NC	NC	NC	NC	2.7	4.5	4.9	3.4	NC
JN8	NC	NC	3.3	2.1	NC	NC	NC	NC	2.6	NC
JN47	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
JN61	NC	NC	NC	NC	NC	NC	3.5	4.0	NC	NC
JN62	NC	NC	NC	NC	NC	NC	NC	NC	2.7	NC
M2-Rev	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
M2-F1a ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

Table 16b. Chemotactic responses of P. aeruginosa strains towards mucin-associated amino acids at concentrations of 10^{-1} - 10^{-5} M

STRAIN	Glycine					Proline				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	2.4	2.5	2.4	2.2	3.0	3.5	3.7	2.9	2.1
J1385	3.8	4.0	4.7	2.7	2.8	NC	5.1	5.2	4.3	NC
J1375	NC	3.8	4.2	4.0	3.3	2.4	3.9	4.8	2.9	3.4
JN8	NC	4.5	14.3	14.4	4.3	NC	8.7	10.0	4.5	NC
JN47	2.2	2.9	3.7	2.3	NC	2.6	NC	2.4	NC	NC
JN61	4.9	4.9	5.9	5.1	3.1	NC	2.4	7.1	3.7	3.4
JN62	4.5	NC	3.5	NC	8.1	2.1	2.7	2.9	3.7	2.5
M2Rev	NC	2.7	3.6	3.0	2.9	NC	3.1	3.8	2.5	2.2
M2Fla ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

STRAIN	Serine					Threonine				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	2.7	6.1	5.2	2.2	NC	NC	NC	NC	NC
J1385	3.3	3.9	4.7	3.5	NC	NC	NC	2.9	2.5	2.7
J1375	NC	3.7	4.0	3.4	3.3	2.9	2.2	3.1	3.0	NC
JN8	NC	8.1	19.2	15.7	10.9	NC	4.7	6.6	NC	4.3
JN47	5.5	NC	6.1	4.2	NC	3.2	5.5	NC	72.	2.7
JN61	2.0	3.9	3.8	2.7	NC	NC	2.1	4.4	NC	3.0
JN62	1.9	2.9	2.2	NC	NC	NC	2.8	3.7	NC	NC
M2Rev	NC	2.8	3.0	2.7	NC	NC	NC	2.3	NC	NC
M2Fla ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing an amino acid to accumulation of bacteria in a control capillary containing no chemoattractant. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. NC = no chemotaxis (ie <2.0).

c. M2Fla⁻ = non-flagellate control.

d. WR-5 = non-chemotactic control.

Table 17. Chemotactic responses of *P. aeruginosa* strains towards mucin-associated sugars at concentrations of 10^{-1} - 10^{-5} M.

STRAIN	L-fucose					D-galactose				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	NC	NC	2.1	2.5	2.4	3.7	5.7	6.9	15.1
J1385	3.0	5.8	2.2	2.4	2.6	NC	NC	3.8	2.2	3.5
J1375	NC	NC	NC	2.8	NC	NC	NC	NC	NC	NC
JN8	NC	NC	NC	3.6	5.4	NC	2.3	3.3	NC	NC
JN47	NC	2.1	2.8	NC	NC	NC	2.4	3.0	NC	NC
JN61	NC	NC	NC	NC	NC	5.6	NC	NC	NC	NC
M2Rev	NC	3.1	2.8	2.9	NC	NC	NC	NC	NC	NC
M2Fla ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

STRAIN	GalNAc					GlcNAc				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
PA01	NC	NC	NC	NC	NC	NC	2.3	NC	NC	NC
J1385	NC	3.4	NC	NC	NC	3.5	4.5	3.2	3.0	2.7
J1375	NC	NC	NC	NC	NC	3.0	2.7	NC	NC	NC
JN8	NC	2.1	2.3	2.3	4.2	NC	NC	3.5	4.3	3.5
JN47	NC	2.8	2.1	NC	NC	NC	NC	2.4	2.8	NC
JN61	NC	NC	NC	NC	2.3	NC	NC	NC	NC	NC
JN62	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
M2Rev	NC	4.7	NC	NC	NC	2.1	2.1	NC	2.4	2.2
M2Fla ⁻	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
WR-5	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

a. The chemotactic response is the ratio of accumulation of bacteria (viable count) in capillaries containing a sugar to accumulation of bacteria in a control capillary containing no chemoattractant. A response value >2.0 is considered meaningful (Moulton & Montie, 1979).

b. NC = no chemotaxis (ie <2.0).

c. M2Fla⁻ = non-flagellate control.

d. WR-5 = non-chemotactic control.

5.9 MOTILITY STUDIES

a) Effect of anti-flagellar Mab on chemotaxis/motility

Experiments were carried out to assess the effect of an anti- flagellar Mab (type-b) on chemotaxis of *P. aeruginosa* strains, PAO1 and J1385 (both flagellar type-b), towards porcine gastric mucin (Figure 64). In the absence of antibody both strains showed a peak chemotactic response to mucin after 15 min (PAO1) and 30 min (J1385). However, in the presence of antibody no meaningful chemotactic response was observed, indicating inhibition of motility and consequently chemotaxis of the two organisms.

b) Motility assay

Migration of non-mucoid and mucoid *P. aeruginosa* strains in semi- solid motility agar was investigated (Table 18). Considerable variation in motility, indicated by the diameter of the spreading colony, was noted. The five non-mucoid, colonising strains of *P. aeruginosa*, isolated from CF patients with no previous history of CF, showed good motility in the semi-solid agar. The difference between motility of these colonising strains and all other clinical strains of *P. aeruginosa* was significant (Student *t* test, $P < 0.05$). However, the motility difference between all the non-mucoid and mucoid clinical strains was not significant.

Motility of non-mucoid *P. aeruginosa* strains in semi-solid minimal media containing various concentrations of porcine gastric mucin as the carbon/nitrogen source was also observed (Table 19), and indicated that mucin was able to support growth and advancement of the spreading colonies of *P. aeruginosa*.

5.10 GROWTH OF *P. AERUGINOSA* ON MUCINS

In a more comprehensive study involving both non-mucoid and mucoid strains of *P. aeruginosa*, mucin was shown to support the growth of *P. aeruginosa*, providing both nitrogen and carbon source requirements and support for

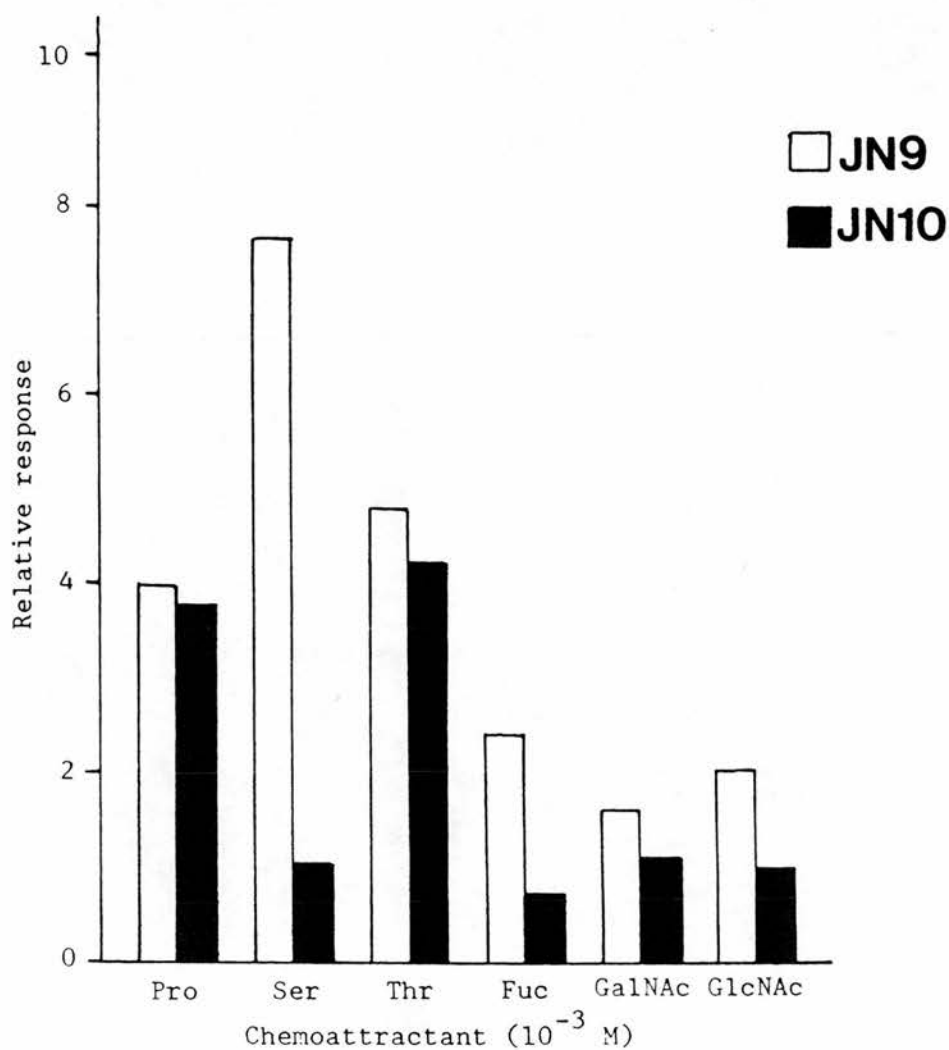


Figure 63. Chemotactic responses of isogenic non-mucoid (JN9) and mucoid (JN10) *P. aeruginosa* strains toward amino acid and sugars. The relative response is the ratio of the mean accumulation of bacterial CFU in duplicate capillaries containing a potential chemoattractant to the accumulation of bacterial CFU in control capillaries containing CM alone. A response >2.0 is considered to indicate chemotaxis.

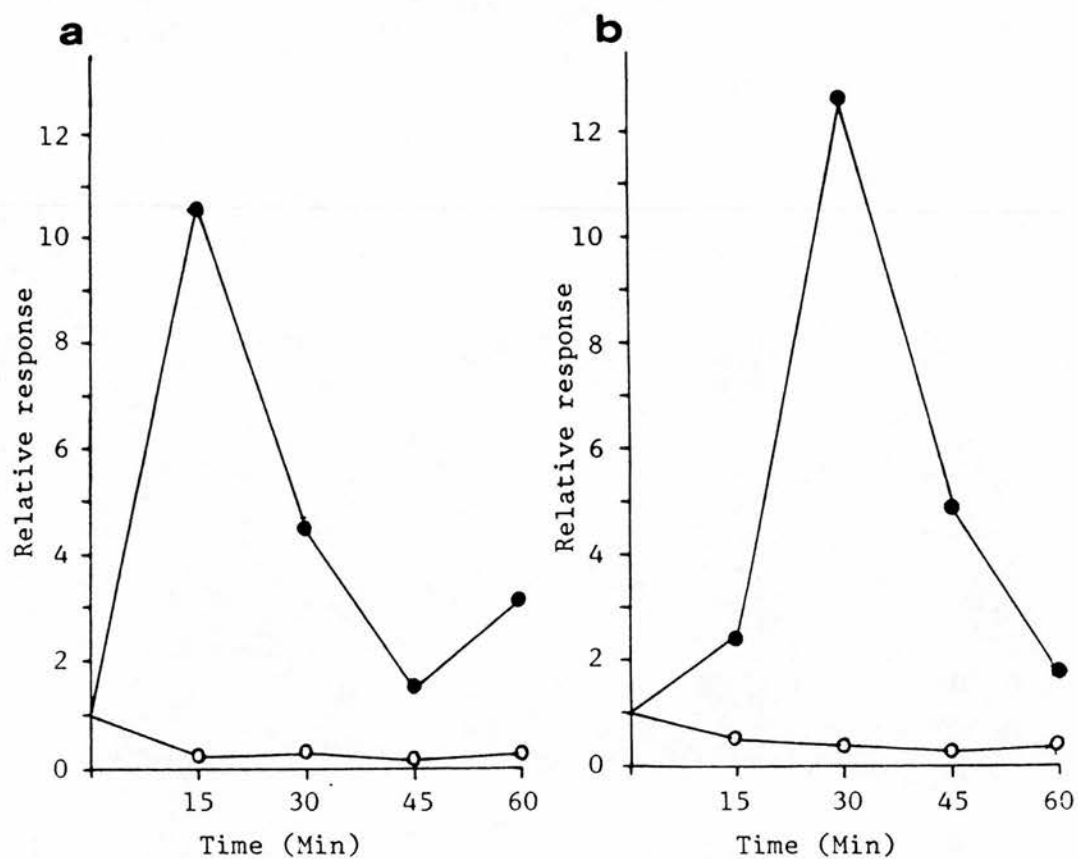


Figure 64. Relative chemotactic responses of two *P. aeruginosa* strains - PAO1 (a) and J1385 (b) towards porcine gastric mucin (100 $\mu\text{g/ml}$) in the absence (●—●) and presence (O—O) of anti-*P. aeruginosa* flagellar type-b Mab (1:40 in the bacterial suspension).

Table 18. Motility of clinical (from patients with CF) and non-clinical *P. aeruginosa* strains in semi-solid agar as determined by diameter (mm) of the spreading colony, after 24 h.

STRAIN	Diameter of spreading colony (mm)
JN8* ^b	18
JN47*	20
JN61*	25
JN62*	16
J1385*	35
J1375	25
PAO1	23
JN9	14
JN11	10
JN28	14
JN37	38
JN41	2
JN43	14
JN44	30
JN45	2
JN46	0
JN48	2
JN49	2
JN52	0
JN3 (Muc) ^a	2
JN10 (Muc)	6
JN12 (Muc)	10
JN16 (Muc)	12
JN20 (Muc)	2
JN36 (Muc)	6
JN50 (Muc)	2
JN51 (Muc)	4
JN53 (Muc)	0
PAO568 (Muc)	6
PAO578 (Muc)	20
M2Rev ^d	16
M2Fla ^{-c}	2
WR-5 ^e	10

a) Muc = mucoid strain.

b) * = early colonising, non-mucoid strains from patients with CF.

c) M2Fla⁻ = non-flagellate control.

d) M2Rev = motile revertant of M2Fla⁻.

e) WR-5 = non-chemotactic mutant

Table 19. Motility of P. aeruginosa strains from patients with CF, and non-clinical strains in semi-solid minimal media (MM) supplemented with mucin as the carbon/nitrogen source.

STRAIN	MM + mucin 1% w/v	MM + mucin 0.5% w/v	MM + mucin 0.1% w/v	MM only
PAO1 ^a	15 ^b	21	17	-
J1385	16	18	14	-
J1375	14	10	9	-
JN8	13	15	10	-
JN47	12	9	5	-
JN61	15	9	5	-
JN62	15	14	11	-

a. PAO1 = classic genetic strain; J1375 = environmental isolate. All other strains isolated from patients with CF.

b. Motility determined by diameter (mm) of the spreading colony.

Table 20. Growth of P. aeruginosa strains on minimal media containing mucin (1% w/v) as a nitrogen, carbon, and nitrogen/carbon source.

STRAIN	Mucin as nitrogen source	Mucin as carbon source	Mucin as nitrogen/carbon source	Nutrient agar
PAO1	+++ ^a	+	++	++++
J1385	+++	++	++	++++
J1375	+++	++	++	++++
JN8	+++	++	++	++++
JN9	+++	+	+	++++
JN10 (Muc) ^b	++	+	+	++++
JN20 (Muc)	+++	++	++	++++
JN36 (Muc)	++	++	+	++++
JN47	++	+	+	++++
JN61	++	++	++	++++

a. Growth measured relative to growth on nutrient agar:
++++ (maximal); +++ (good); ++ (Moderate); + (slight).

b. Muc = mucoid strain.

production of the mucoid phenotype (Table 20).

Growth studies with the individual sugars of mucin (L-fucose, D-galactose, GalNAc, GlcNAc and NANA) as carbon sources, revealed that only GlcNAc was able to support growth of *P. aeruginosa*. (Glucose as the positive control was also included in the study.

5.11 ADHERENCE OF *P. AERUGINOSA* TO MUCINS

Two novel methods were developed to examine adherence of *P. aeruginosa* to mucin. The *P. aeruginosa* strains included in these studies corresponded to those used for the chemotaxis experiments plus two non-mucoid colonising strains, JN83 and JN89, isolated from patients with CF.

a) Adherence to mucin monolayers

Adherence of *P. aeruginosa* to both porcine and CF mucin monolayers was observed with each of the *P. aeruginosa* strains examined. Initial experiments, performed with porcine gastric mucin, showed *P. aeruginosa* cells to be associated with, and often entangled in, a highly aggregated mucin matrix, (Figures 65a-c & 66). When these experiments were repeated with mucin (gel fraction) purified from the tracheobronchial secretions of a CF patient, *P. aeruginosa* adhesion was again observed. CF mucin appeared in an aggregated form attached to the bacterial cell surface (Figure 67a-d). Attachment of mucin fragments to flagella was also observed (Figure 67c). Many of the bacterial cells observed in the monolayers possessed flagella, and phase-contrast microscopy confirmed that all the bacteria, except strain M2Fla⁻, were initially motile when incubated in the presence of mucin. As a control for random association or simple entanglement between mucin and bacterial cells, cultures of *E. coli* K12, *Pr. mirabilis* and *K. aerogenes* were added to mucin, and monolayers prepared. In contrast to *P. aeruginosa* only an occasional bacterial cell was observed in these monolayers, and those observed were not

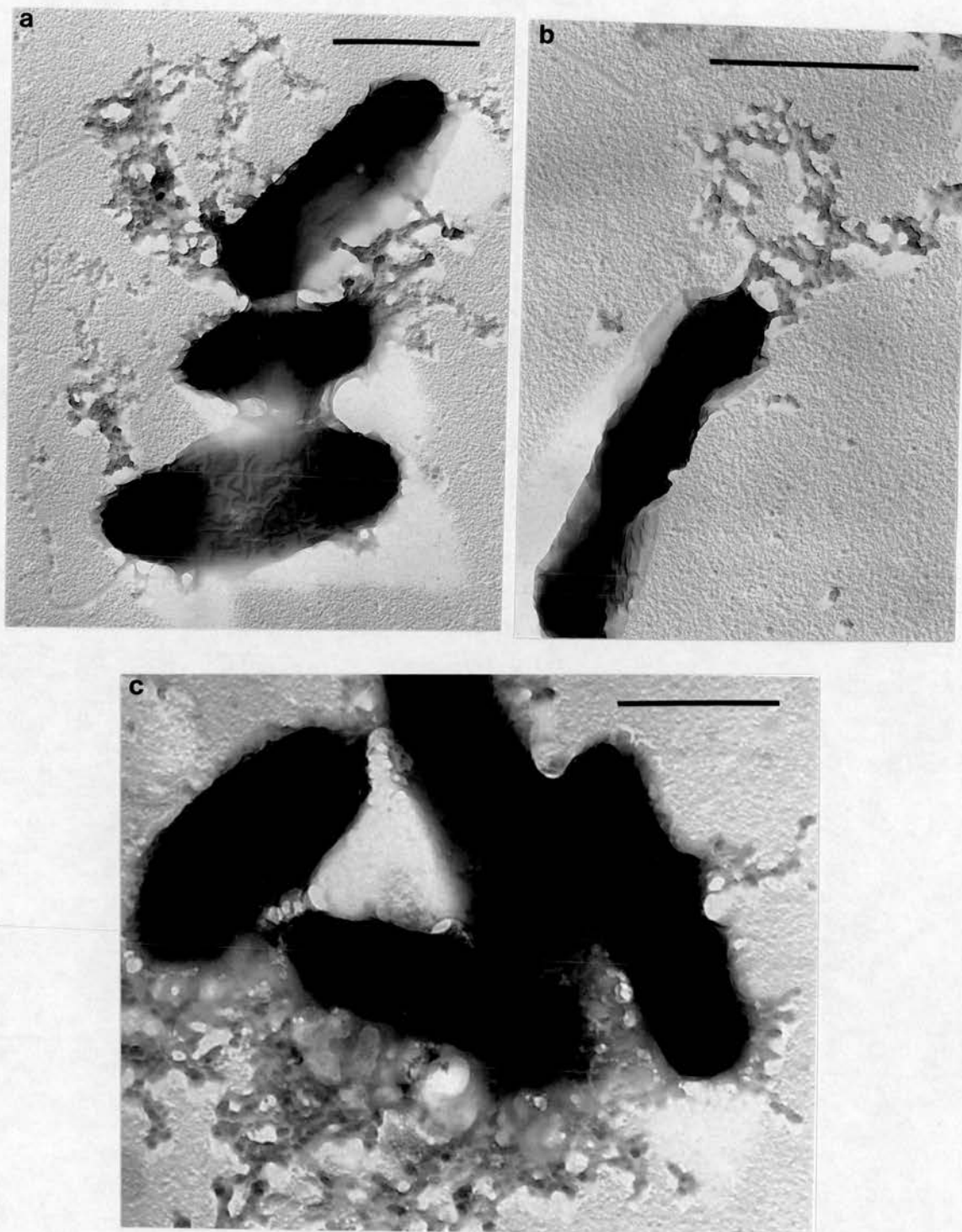


Figure 65a-c). Electron micrographs of *P. aeruginosa* strains JN8, J1385, and JN61 in association with purified porcine gastric mucin (0.05 $\mu\text{g}/\text{ml}$) spread in BAC monolayer stained with uranyl acetate and subjected to unidirectional shadowing with platinum. Bar, 1 μm .

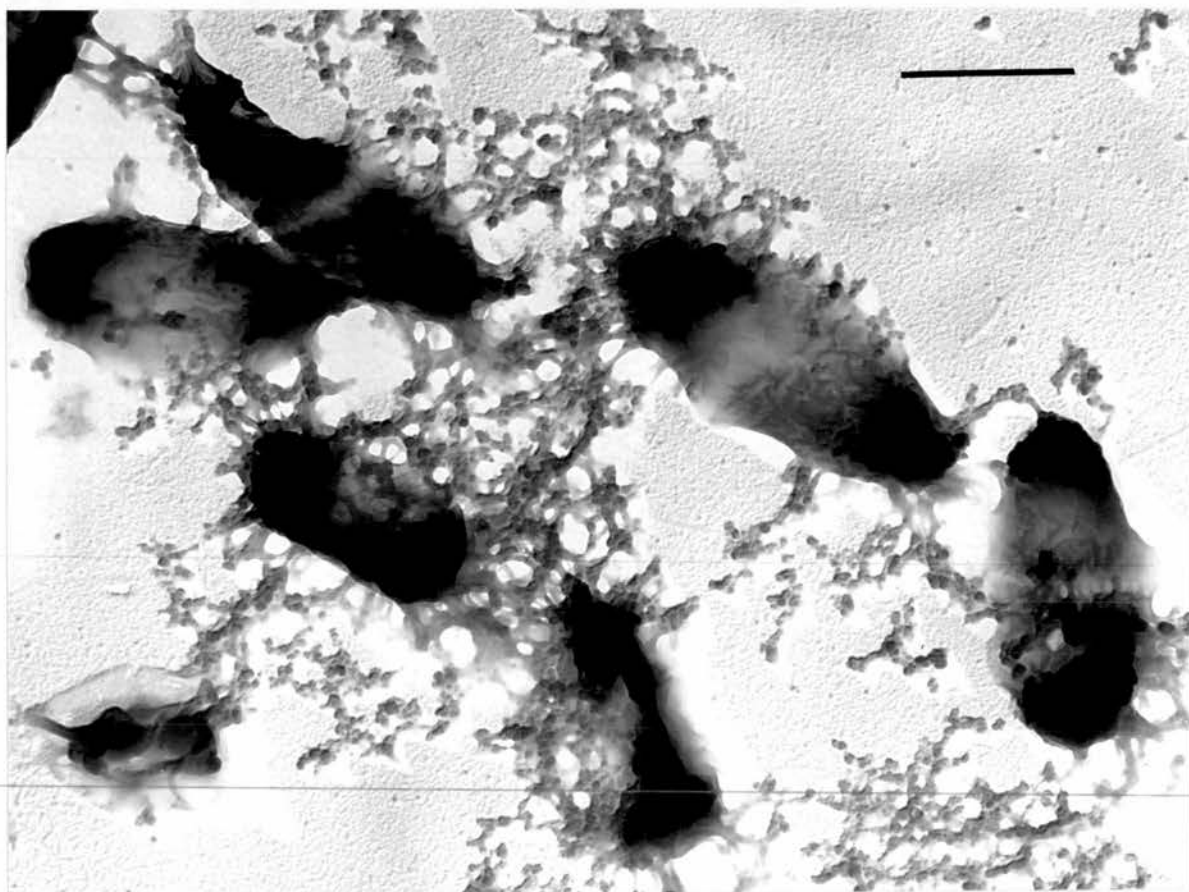


Figure 66. Electron micrograph of *P. aeruginosa* strain JN62 in association with purified porcine gastric mucin (0.05 $\mu\text{g}/\text{ml}$) spread in BAC monolayer stained with uranyl acetate and subjected to unidirectional shadowing with platinum. Bar, 1 μm .

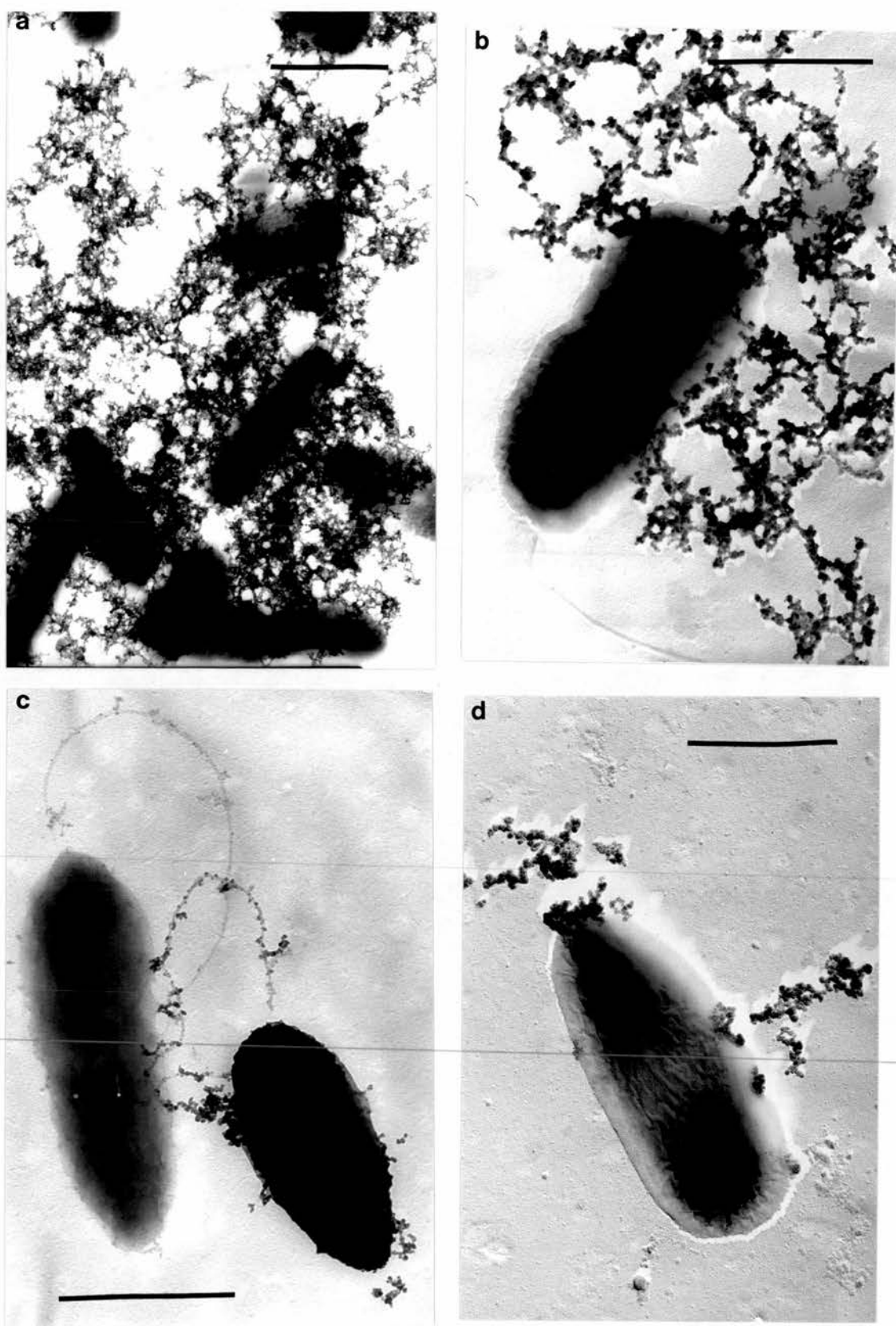


Figure 67a-d). Electron micrographs of *P. aeruginosa* strains WR-5, J1385, J1375, and JN62 in association with purified tracheobronchial mucin from a CF patient showing adhesion of mucin aggregates to bacterial surfaces. Monolayers were stained with uranyl acetate and subject to unidirectional shadowing with platinum. Bars, 1 μ m.

attached to mucin (Figure 68a & b). In these control monolayers with non-adherent bacteria, the mucin appeared as lightly stained linear filaments similar in appearance to mucin in the absence of bacteria.

b) Microtitre ELISA for measurement of *P. aeruginosa* adhesion to mucin

Interaction and association of *P. aeruginosa* cells with mucin were clearly demonstrated in the electronmicrographs. Transmission electron microscopy of bacteria in mucin monolayers was, however, not suitable for screening many *P. aeruginosa* strains with a number of different mucins. As an alternative, a microtitre ELISA technique incorporating one of the anti-*P. aeruginosa* Mabs described earlier (see Chapter 3) was used to measure the adherence of twelve *P. aeruginosa* strains to mucins bound to the wells of polystyrene microtitre plates.

In preliminary experiments commercially available porcine gastric mucin was used to coat wells of microtitre plates. The concentration of mucin required to optimally coat wells, and the optimal concentration of bacteria added to wells were determined. A mucin coating concentration of 100 µg/ml was shown to give optimal sensitivity, yielding a maximal difference in OD between mucin coated wells and background controls. A bacterial concentration of 10^7 cells/ml showed maximal measurable binding to mucin coated wells and minimal non-specific binding to background control wells. At bacterial concentrations greater than 10^8 cells/ml, significant binding to uncoated wells was evident. The effect of increasing mucin concentration on adherence of six strains of *P. aeruginosa* is shown in Figure 69. In general, measurable adherence of *P. aeruginosa* cells increased with increasing concentration of mucin, optimal binding occurring at between 100-200 µg/ml of mucin. Adherence of twelve *P. aeruginosa* strains to porcine mucin was observed (Figure 70).

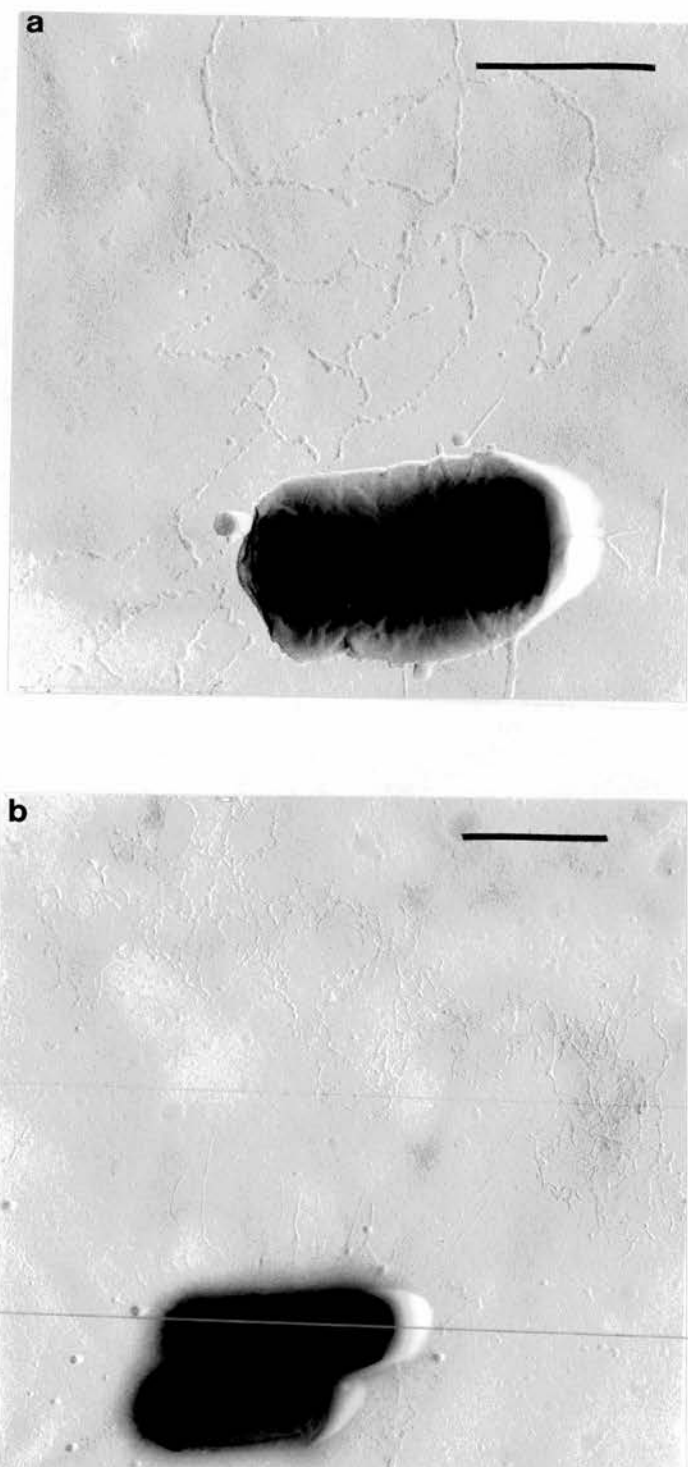


Figure 68. Electron micrographs of one of the few bacterial cells observed when *E. coli* K-12 (a) and *K. aerogenes* (b) were investigated in mucin monolayers. No direct mucin-bacterium associate is evident, and the mucin appears as lightly stained linear filaments. Monolayers were stained with uranyl acetate and subjected to unidirectional shadowing with platinum. Bars 1 μm .

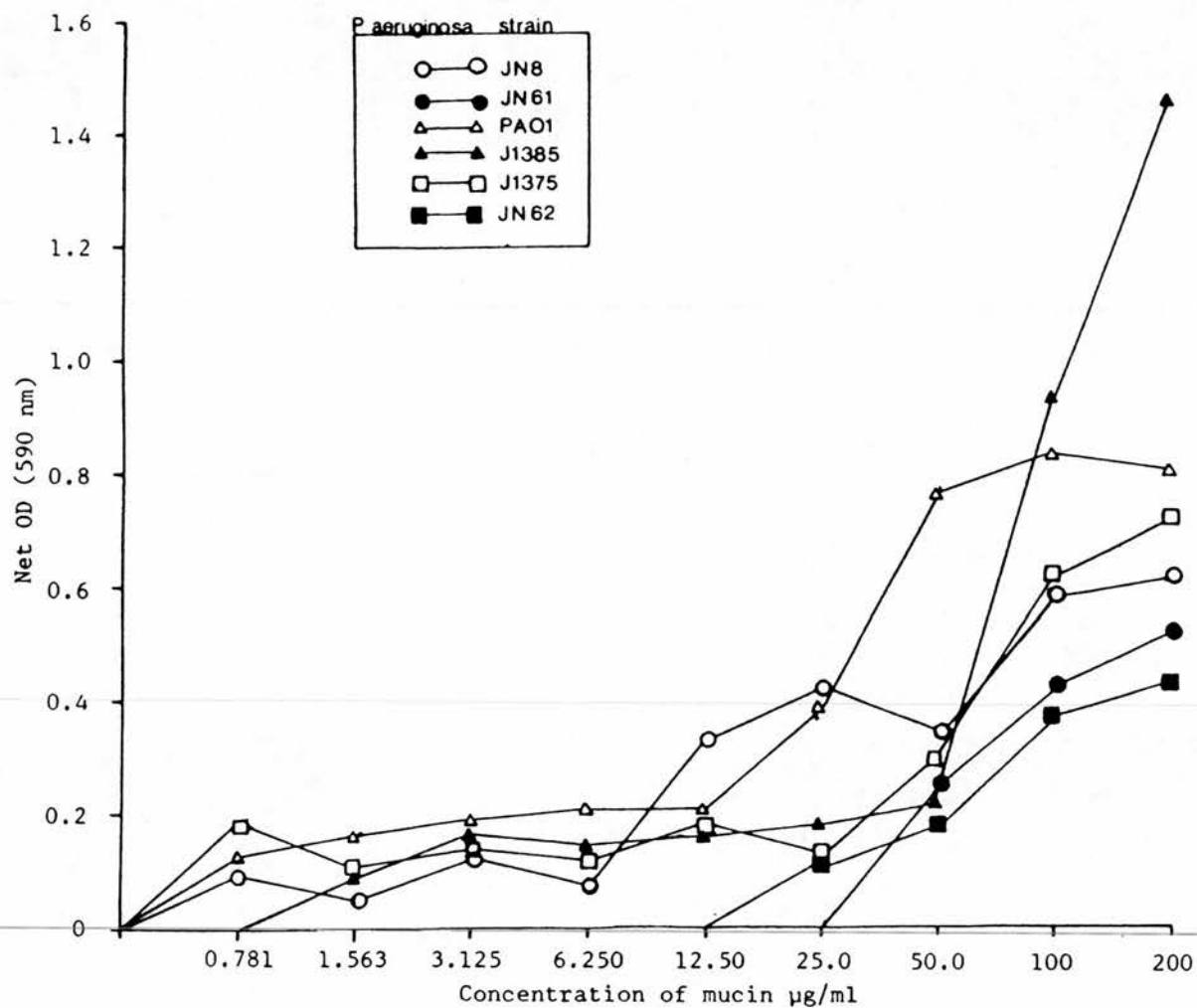


Figure 69. Effect of increasing mucin (porcine-gastric) concentration on the adherence of 6 *P. aeruginosa* strains. Adherence measured by ELISA (OD 590 nm) incorporating Mab (1:500). OD values represent the mean from duplicate experiments in which the mean OD from triplicate wells is expressed after subtraction of negative background controls.

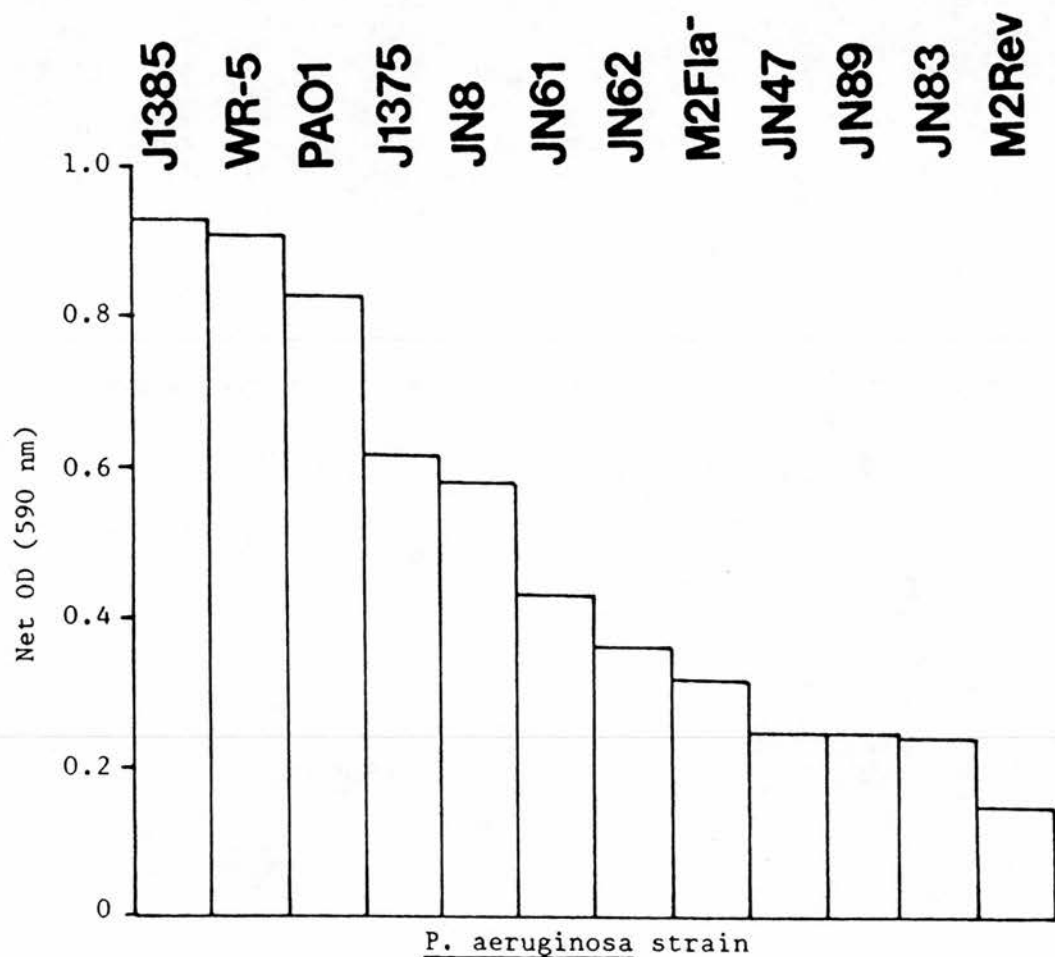


Figure 70. Adherence of 12 *P. aeruginosa* strains to porcine gastric mucin (100 μ g/ml). Adherence measured by ELISA (OD 590 nm) incorporating anti-*P. aeruginosa* LPS Mab (360.7). OD values represent the mean from duplicate experiments in which the mean OD from triplicate wells is expressed after subtraction of negative background controls.

In subsequent experiments adhesion of *P. aeruginosa* strains to a number of human derived mucins was investigated (Table 21). Strain dependent adhesion to most of the mucin preparations was observed, particularly to the small salivary mucins and CF gel glycopeptide fragments. Measurable adhesion of all the *P. aeruginosa* strains to fraction D of the fractioned CF gel mucin was observed. On the basis of the results obtained, there appeared to be no significant correlation between adhesion and chemotaxis to particular mucins, eg examination of results for the fractioned CF gel mucin indicated that fraction B was the most effective chemoattractant whilst adhesion of *P. aeruginosa* to fraction D was most evident.

5.12 EFFECT OF SUGARS ON ADHERENCE

The effect of the mucin sugars L-fucose, D-galactose, GalNAc, GlcNAc and NANA, on *P. aeruginosa* adherence to mucins was investigated. The effect of the different mucin sugars on adhesion of six *P. aeruginosa* strains to CF mucin gel glycopeptides was strain dependent (Table 22). Overall inhibition of bacterial adhesion to mucin was most evident with GlcNAc and NANA, although fucose mediated the greatest inhibitory effect on adhesion of PAO1 and J1385.

Table 21. Adherence^a of P. aeruginosa strains to purified mucins

	<u>P. aeruginosa</u> strain											
	PA01	J1385	J1375	JN8	JN47	JN61	JN62	JN83	JN89	M2Rev	M2Fla ⁻	WR-5
Mucin 100 µg/ml												
CF sol	0.40 ^b	0.66	0.11	0.50	0.19	0.64	0.53	0.17	0.14	0.18	0.16	0.58
CF gel	0.35	0.22	0.45	0.22	0.49	0.39	0.25	0.31	0.19	0.28	0.24	0.50
CF gel Fraction A	0.57	0.12	0.19	- ^c	0.21	0.55	0.47	-	0.80	0.90	-	0.60
CF gel Fraction B	0.35	0.19	0.18	0.18	0.13	0.33	-	0.90	0.10	0.60	0.19	0.49
CF gel Fraction C	0.35	0.25	0.60	-	0.13	0.28	-	0.60	0.10	0.70	0.70	0.48
CF gel Fraction D	0.49	0.16	0.21	0.44	0.24	0.45	0.11	0.17	0.31	0.18	0.26	0.72
Chronic bronchic	0.67	0.64	0.45	0.13	0.15	0.46	0.11	0.10	0.21	0.47	0.29	1.07
Normal-large salivary	0.45	0.66	0.15	0.32	0.10	0.34	0.29	0.38	0.33	0.38	0.34	0.79
Normal-small salivary	1.18	1.36	1.27	0.95	1.17	0.87	1.15	0.24	0.83	1.36	0.72	1.43
Asthmatic	0.38	0.40	0.34	0.14	0.30	0.31	0.28	0.41	0.17	0.22	-	0.76
Gel glycopeptides	1.00	1.27	0.47	0.68	0.71	0.81	0.80	0.53	0.51	0.68	0.65	1.33
Sol glycopeptides	0.55	0.77	0.30	0.16	0.51	0.81	0.11	0.27	0.23	0.16	0.14	0.68

a. Adherence measured by ELISA (OD 590 nm) incorporating anti-P. aeruginosa LPS Mab (360.7).

b. OD results represent the mean from duplicate experiments in which the mean OD from triplicate determinations is expressed after subtraction of negative background controls. An OD>0.1 was classed as significant in terms of adherence.

c. - = OD<0.1

Table 22. Effect of mucin associated sugars on adherence^a of P. aeruginosa to mucin gel glycopeptides (100 µg/ml)

STRAIN	UNINHIBITED	Sugar Inhibitor (100 mM)			
		L-fucose	D-galactose	GalNAc	GlcNAc
PA01	1.10 ^b	0.71 (40%) ^c	1.20 (-)	0.87 (26%)	0.80 (32%)
J1383	0.45	0.19 (57%)	0.38 (15%)	0.42 (5%)	0.21 (53%)
JN8	0.41	0.56 (-)	0.38 (8%)	0.34 (17%)	0.19 (53%)
JN47	0.86	0.65 (25%)	0.79 (9%)	0.59 (32%)	0.27 (69%)
JN61	0.99	0.94 (5%)	0.96 (3%)	0.92 (7%)	0.46 (53%)
JN62	0.54	0.54 (-)	0.50 (8%)	0.46 (16%)	0.16 (70%)

a. Adherence measured by ELISA (OD 590 nm) incorporating anti-P. aeruginosa LPS Mab.

b. OD (590 nm) results represent the mean from duplicate experiments in which the mean OD from triplicate determinations is expressed after subtraction of negative background controls.

c. Percentage inhibition of adherence compared with uninhibited control.

DISCUSSION

CHAPTER 1

MUCINOPHILIC PROPERTIES OF *P. AERUGINOSA*

In contrast to the considerable accumulation of information on the adaptation and pathogenesis of mucoid *P. aeruginosa*, little attention has been directed to the bacterial and host factors which may contribute to the initial stages of asymptomatic colonisation with non-mucoid *P. aeruginosa* which, arguably, forms the microbial reservoir for the subsequent emergence of mucoid variants. For example, why do some CF patients become colonised by *P. aeruginosa* whilst other patients of the same age and attending the same centre remain free from colonisation for long periods? Exposure to *P. aeruginosa* strains more able to colonise the CF lung than others, may increase the risk of colonisation, whilst some CF patients may be more susceptible to microbial colonisation because of certain genetic or environmental factors (Govan & Glass, 1990).

One of the aims of this thesis was to examine the mucinophilic properties of non-mucoid *P. aeruginosa* and the role such characteristics may contribute to the initial stages of pulmonary colonisation in patients with CF. The affinity of *P. aeruginosa* for mucin was investigated since mucus glycoproteins (mucins) are the major macromolecular components of the mucosal layer lining the respiratory tract (Rose, 1988) and are produced in excessive amounts in the respiratory secretions of patients with CF (Chace *et al*, 1985).

1.1 CHEMOTAXIS OF *P. AERUGINOSA* TOWARDS MUCIN

Many motile bacteria have the capacity to move towards nutrients (chemotaxis), thus entering a more favourable environment and perhaps enhancing their association with a host surface (Finlay & Falkow, 1989). Allweiss

et al (1977) reported that chemotactic mechanisms promoted the association of *E. coli*, vibrios and salmonellae with rabbit mucosal tissue. In a series of subsequent and related investigations Freter and colleagues (Freter *et al*, 1979; Freter *et al*, 1981a & b; Freter & O'Brien, 1981a & b) also observed chemotaxis of *Vibrio cholerae* towards mucosal slices and a pepsin digest of rabbit mucosal scrapings. Uhlman & Jones (1982) demonstrated that a diffusible attractant released from HeLa cells greatly enhanced the collision frequency between *S. typhimurium* and the epithelial cells. A more recent study of the factors required by *Salmonella typhi* for invasion of HeLa cells suggested that flagella, motility and chemotaxis were all necessary (Liu *et al*, 1988).

In addition to the chemotaxis studies of *V. cholerae* and its subsequent association with mucosal surfaces, a number of other studies also support the hypothesis that chemotaxis towards mucin or secretions of mucosal surfaces is a potentially important bacterial virulence mechanism. Chemotaxis of *Treponema hyodysenteria* (Kennedy *et al*, 1988) and *Campylobacter jejuni* (Hugdahl *et al*, 1988) towards porcine gastric mucin have been demonstrated, whilst Hazell *et al*, 1986) speculated that the association of *Helicobacter pylori* (*Campylobacter pyloridis*) with the gastric mucosa may be aided by chemotactic movement of the organism into the gastric pits and toward the intracellular junctions from which potential nutrients diffuse.

In this thesis the chemotactic response of *P. aeruginosa* towards a number of different mucin preparations was examined (Tables 4-11). Chemotaxis towards both CF and non-CF mucins was observed and represents the first documented report of the chemotaxis of *P. aeruginosa* towards mucin. The fact that chemotaxis towards a number of different mucin preparations was demonstrated suggests that the intrinsic composition and structure of CF mucin is not exclusively chemoattractive for *P. aeruginosa*. Comparisons of the carbohydrate compositions cannot demonstrate structural differences between CF and non-

CF mucins as the analyses reflect an averaging of hundreds of oligosaccharide chains which may differ in size, core type and structure (Rose, 1988). However, Houdret *et al*, 1989 reported that the increased acidity and the decreased proportion of neutral glycopeptides in the respiratory secretions of both CF and non-CF individuals was related to infection with *P. aeruginosa*. The high lipid content of respiratory mucins in CF patients is also a result of infection with *P. aeruginosa* and not directly related to infection (Houdret *et al*, 1986). The CF mucin used in the chemotaxis and adherence experiments was isolated from the tracheobronchial secretions of a female CF patient already colonised by *P. aeruginosa*. Chemotaxis of *P. aeruginosa* to the fractions of CF gel mucin as well as the CF gel glycopeptides also indicates that the chemotaxis is not specific for a particular composition or structural domain of mucin. The amount of sialic acid in the individual fractions of CF mucin decreases from fraction A to fraction D, with a concomitant decrease in acidity (Dr D.J. Thornton, pers. comm.).

Chemotaxis of *P. aeruginosa* towards the major amino acids and sugars of mucin was demonstrated (Tables 16a & b and 17). The degree of chemotaxis towards the different components appeared to be strain dependent. Previous studies have indicated that *P. aeruginosa* is responsive to a wide range of attractants including glucose, organic acids and many of the twenty commonly occurring amino acids (Moulton & Montie, 1979; Craven & Montie, 1985). In this thesis, the results obtained with the mucin-associated amino acids and sugars showed that the chemotactic responses towards the amino acids were generally greater than those obtained for sugars. These observations correspond to those made by other investigators who also demonstrated that *P. aeruginosa* was highly responsive towards amino acids and less strongly responsive towards glucose and organic acids (Moench & Konetzka, 1978; Moulton & Montie, 1979). In the study of Moulton & Montie (1979) the authors observed a lack of response to glucose when *P. aeruginosa* was grown in succinate, malate or citrate containing media and concluded that the results

were consistent with the induction, by glucose, of a glucose binding protein reported by Stinson *et al* (1976), which also functions as a chemoreceptor (Stinson *et al* (1977). These findings may account for the generally low chemotactic responses observed for the mucin sugars. Prior to use in the chemotaxis assays *P. aeruginosa* was grown in minimal media, containing citrate as the carbon source. Optimum chemotaxis towards some or all of the mucin sugars may be dependent on inducible chemoreceptors.

No effect of the carbon source on *P. aeruginosa* chemotaxis towards amino acids was observed by Moulton & Montie (1979). However, Craven & Montie (1985) showed that chemotaxis towards several amino acids is subjected to control by nitrogen availability and that this regulation is probably expressed at the level of chemoreceptors or transducers. The authors concluded that chemotaxis towards amino acids may be a mechanism of nitrogen acquisition by *P. aeruginosa*. In an earlier study, Kay & Gronlund (1969) reported that twenty common amino acids can be utilized by *P. aeruginosa* as sole nitrogen sources and that transport of most of the amino acids is maintained or increased under conditions of nitrogen starvation. The ability of *P. aeruginosa* to respond to the amino acids of mucin, as demonstrated in this thesis, may play a role in the scavenging of nitrogen within the environment of the CF lung. In addition, mucin was shown to support the carbon and nitrogen source requirements of *P. aeruginosa*.

The mechanism by which *P. aeruginosa* exhibits chemotaxis to mucins, which are high molecular weight glycoproteins (Rose, 1988) is uncertain. The bacterial chemoreceptors cited in the literature appear to be located in the periplasmic space or cytoplasmic membrane (Brass, 1986), although chemotaxis may be mediated by a mechanism involving binding of mucin to a transmembrane outer-membrane receptor protein. Alternatively, chemotaxis to mucin may involve initial degradation of mucin into glycopeptide fragments or individual amino acid and sugar components. Hancock & Nikaido (1978)

reported that protein F formed a large diffusion pore allowing the diffusion of polysaccharides with a *Mr* of up to 9,000. The authors hypothesized that such large pores would permit entry of large peptides into the periplasmic space, rendering them susceptible to peptidases, whereas any extracellular proteases may be involved in the initial processing of proteins in the environment.

Mucins have a general structure consisting of a protein core surrounded by a sheath of carbohydrate side chains, as well as non-glycosylated regions (naked regions) which are susceptible to proteolytic digestion (Clamp *et al*, 1978; Bara *et al*, 1988). Pronase digestion of mucin results in the formation of mucin glycopeptides (Scawen & Allen, 1977; Rose *et al*, 1984). In this thesis, proteolytic digestion of CF mucin and normal salivary mucin into glycopeptide fragments prior to their use in chemotaxis assays did not significantly affect the chemotactic responses of *P. aeruginosa* compared to the non-pronase treated mucins (Tables 13 & 14). Fragmentation of the mucins facilitating transport uptake and provision of individual chemoattractants, may have been expected to enhance the chemotactic response of *P. aeruginosa*. The degraded mucins are likely to consist of glycosylated, proteolytic resistant glycopeptide fragments as well as a mixture of peptides and amino acids. Similarly, the response to CF mucin gel glycopeptides (from which the degraded proteins have been removed) did not significantly alter the chemotactic response of *P. aeruginosa* (Table 12).

In the CF lung, proteolysis of mucin by proteases from *P. aeruginosa* and neutrophils (Poncz *et al*, 1988) could explain how mucin fragments and components would be readily available *in vivo* to act as chemoattractants and nutritional sources for *P. aeruginosa*. Indeed, Rose *et al* (1987) suggested that the smaller size of CF mucins compared to normal and asthmatic mucins was caused by bacterial or inflammatory cell proteases present in CF sputum. They and others (Houdret *et al*, 1989) have postulated that bacterial glycosidases may also be responsible for degradation of mucins in patients with CF. An

extracellular neuraminidase from a *P. aeruginosa* strain isolated from CF has been reported and shown to hydrolyze CF mucin (Leprat & Michel-Briand, 1980). The mucin degrading exoglycosidases produced by oral streptococci (van der Hoeven *et al* 1990) may also provide a ready source of chemoattractants *in vivo* for chemotaxis of *P. aeruginosa*. In the case of *V. cholerae* a mucinase complex has been reported, and may facilitate penetration of the mucus barrier by the organism (Stewart-Tull *et al*, 1986).

The exposure and release of individual amino acids by proteolytic degradation of mucin may influence the degree of chemotactic responses of individual strains. The presence of proteolytic resistant regions within the mucin may reduce the release of serine, proline, threonine, alanine and glycine which are predominant in, but not exclusive to these regions (Scawen & Allen, 1977; Clamp *et al*, 1978; Rose, 1988). However, the naked regions of mucin, un-sheathed by oligosaccharides are easily accessible for proteolytic attack. In addition, *in vivo*, amino acids may also be made available because of proteolysis of other proteins and glycoproteins such as the 65-kilodalton protein reported by Ringler *et al*, 1988, or immunoglobulins and lacto-transferrin, with which respiratory mucin is also associated *in vivo* (Houdret *et al*, 1989).

Since human tracheobronchial mucins typically contain 80-87% w/v carbohydrate (Rose, 1988) the sugar components are likely to play an important role in the chemotaxis of *P. aeruginosa* towards mucin. Indeed, the sugars fucose and NANA are invariably in a terminal position (Clamp *et al*, 1978) and would therefore be likely candidates for chemoattractants. In this study, a strain dependent response towards fucose was observed. In a study of the chemotaxis of *C. jejuni* only fucose of the different mucin sugars tested was identified as a chemoattractant (Hugdahl *et al*, 1988). In this thesis, NANA, which has been reported to be an adhesin for *P. aeruginosa* (Vishwanath & Ramphal, 1985), did not act as a chemoattractant for any of the *P. aeruginosa* strains tested.

NANA is a relatively strong acid in biological systems; however, the failure of NANA to act as a chemoattractant cannot be explained by a low pH value. Although the buffered preparations of NANA at 10^{-1}M did have a low pH, the other preparations examined (10^{-2} - 10^{-5}M) had neutral pHs.

On a technical note, accumulation of bacteria in the capillary tubes containing mucin, amino acid, or sugar attractants cannot be explained by bacteria being carried in passively by convection, brownian movement or random motility since no accumulation of non-motile or non-chemotactic mutants was observed. The different peak values obtained with individual strains showing a response to a given chemoattractant could result from variation in the number of chemoreceptors present, or in the affinities of those receptors. Chemotaxis tends to operate over a limited range of concentrations, the 'response range' (Adler, 1973). At the low extreme is the threshold concentration, the lowest concentration of attractant in the capillary that gives a detectable response. At the high extreme is the saturation concentration, the attractant concentration in the bacterial suspension above which the bacteria cannot detect a still higher concentration in the capillary (Adler, 1973). The operating range is different for individual attractants eg in the case of strain JN8 for fucose, the range was between 10^{-2} and 10^{-1}M , and for galactose it was between 10^{-4} and 10^{-3}M (threshold and saturation respectively). The lag period before chemotaxis is observed is considered to be the time taken to reduce the concentration of attractant at the mouth of the capillary to just below the saturating concentration. This reduction is brought about by utilization of attractant by the bacteria, in the case of metabolizable attractants, and diffusion of the attractant, eg in the case of strain J1375 and its chemotaxis towards CF mucin (Table 5), the lag period was between 30-45 mins before a meaningful chemotactic response was detected. A reduced response at high concentrations of attractant simply reflects the longer lag periods found for high concentrations: it does not usually represent an inhibition of chemotaxis (Adler, 1973).

The chemotaxis assays showed that *P. aeruginosa* strains isolated from patients with CF as well as standard (eg PAO1 and M2Rev) or environmental (J1375) strains are able to respond to mucin as a chemoattractant. However, it could be speculated that in a given environment (eg the whirlpool source of J1385 and J1375) enhanced chemotaxis would confer an advantage on a potential colonising strain eg J1385. In support of this hypothesis J1385 showed greater chemotaxis than J1375 towards most of the mucin preparations, including the CF gel mucin, and towards the majority of mucin constituents.

Motility of *Treponema* (Kennedy *et al*, 1988) *C. jejuni* (Ferrero & Lee, 1988) and *H. pylori* (Hazell *et al*, 1986) in viscous mucosal preparations have been demonstrated. In this study, motility of non-mucoid *P. aeruginosa* strains in a semi-solid medium containing various concentrations of mucin was observed (Table 19). Motility of a number of *P. aeruginosa* strains was also compared (Table 18): enhanced motility of the initial colonising strains, compared to the other non-mucoid and mucoid strains isolated from patients with CF, was demonstrated. These motility experiments confirm the earlier observations of Luzar & Montie (1985) and Luzar *et al* (1985) who showed that several physiological characteristics associated with invasiveness including chemotaxis and motility, appeared to be selected against after initial colonisation of the lungs by the organism: reduced motility and chemotaxis was particularly associated with R-LPS strains isolated from patients in poor clinical condition (Luzar *et al*, 1985). Since the genetic loci for motility and chemotaxis are in close proximity (Tsuda & Iino, 1983) a mutational event in that region of the chromosome might alter both gene products. Alternatively, altered synthesis of LPS could create envelope instability which could interfere with the secure anchoring of the flagellum (Luzar *et al*, 1985). Some authors have attempted to correlate mucoidy with non-flagellation (Boyce & Miller, 1982b). However, motility of some of the mucoid strains was observed in the semi-solid agar motility experiments and no such correlation was demonstrated in the results of

the study by Luzar *et al* (1985). In this thesis, an experiment was performed to compare chemotaxis of isogenic non-mucoid and mucoid *P. aeruginosa* isolates (Figure 63). A similar response to some of the attractants was observed, although an elevated response to other attractants by the non-mucoid strain was evident, indicating that the mucoid phenotype may inhibit the motility and/or chemotaxis of *P. aeruginosa*.

There have been relatively few reports describing the chemotactic properties and motility of *P. aeruginosa* *in vivo*. However, some investigations have demonstrated that motility or chemotactic ability, or both, are associated with the virulence of *P. aeruginosa*. Strains which were highly chemotactic or motile, or both, had lower 50%-lethal doses when tested in a burned mouse model (Craven & Montie, 1981), and motility-deficient mutants were found to be less virulent than their wild type parents in a burned rat model (McManus *et al*, 1980). Further experiments with the burned mouse model have also demonstrated that decreased virulence is associated with non-flagellated or non-motile mutants of *P. aeruginosa* (Montie *et al*, 1982b; Drake & Montie, 1988). The results presented in this investigation indicate that the ability of motile, chemotactically directed bacteria to quickly penetrate the mucus blanket, across and against the cephalad movement of the mucociliary escalator (albeit reduced in CF) may play an important role in respiratory colonisation of *P. aeruginosa* in patients with CF.

1.2 ADHERENCE OF *P. AERUGINOSA* TO MUCIN

The ability of *P. aeruginosa* to bind to a number of respiratory cell surfaces as well as tracheobronchial mucins was reviewed extensively in the Introduction. In this thesis, the ability of colonising, non-mucoid strains of *P. aeruginosa*, to bind to mucin was further investigated, the development of two methods providing further evidence of the mucinophilic properties of *P. aeruginosa*. Novel application of transmission electron microscopy and BAC monolayers demonstrated *P. aeruginosa* entanglement and adherence to porcine gastric

mucin and purified CF mucin. The observation that individual strains of *E. coli*, *Pr. mirabilis* and *K. aerogenes* did not adhere to mucin agrees with the observations of Vishwanath & Ramphal (1984), who also observed only the occasional binding of non-pseudomonal cells, and concluded that the association between mucin and *P. aeruginosa* is relatively specific.

The commercial preparation of porcine gastric mucin was included in the initial adherence studies conducted in this thesis because of its availability and the fact that it is reported to resemble human tracheobronchial mucin in its carbohydrate, amino acid and sulphate ester composition (Boat *et al*, 1977; Scawen & Allen, 1977). In addition to its mucin content the commercial preparation is likely to be contaminated with cell membranes, proteins, DNA etc (Dr J.K. Sheehan, pers. comm.). However, the *in vitro* studies performed with this preparation are probably more reflective of the pulmonary environment colonised by *P. aeruginosa*, where mucin will be associated with the natural contaminants of the mucosal blanket. Confirmation of an association between *P. aeruginosa* and mucin was provided by the studies with the purified CF mucin preparation.

The observation that both non-motile and non-chemotactic mutants of *P. aeruginosa* showed attachment to mucin indicates that adhesion, in this *in vitro* assay, is not dependent on motility or chemotaxis. The presence of mucin aggregates attached to individual cells of *P. aeruginosa* suggests that the bacterium-mucin interaction is strong enough to overcome the sheer forces as the mucin monolayer is formed. In the presence of *P. aeruginosa*, porcine and CF mucin appeared in a highly aggregated form, or as small fragments decorating the bacterial surface. This is in contrast to the flexible, linear, lightly stained threads observed in the case of mucin alone (also reported by Rose *et al* (1984) and Sheehan *et al* (1986)) and in the presence of non-adherent bacteria, eg *E. coli*. This observation suggests that mucin molecules are degraded or undergo a substantial conformational change in the presence of *P. aeruginosa*.

It has been proposed that adhesion of *P. aeruginosa* to mucin (and other surfaces) is mediated by pili in the case of non-mucoid organisms and alginate in the case of mucoid strains (Ramphal *et al*, 1987). However, examination of the micrographs (Figures 65-67) showing fragments of aggregated mucin attached to various surface points of non-mucoid *P. aeruginosa* did not suggest solely pilus mediated adhesion. It has been reported however, that apparently non-mucoid *P. aeruginosa* strains are able to express alginate antigens on their surface (Pier *et al*, 1986; Anastassiou *et al*, 1987) and may account for some of the adhesion to mucin observed. Alternatively, Marcus & Baker (1985) and Plotkowski *et al* (1989) have suggested that lectins may play a crucial role in forming a bridge between bacterial and host surface polysaccharides, resulting in a firm bond between the two. Plotkowski *et al* (1989) observed that *P. aeruginosa* strains exhibiting high internal lectin titres adhered significantly better to elastase treated mucosa. *P. aeruginosa* has been reported to produce two distinct lectins, PA-I and PA-II, which differ in their carbohydrate-binding specificities (Gilboa-Garber, 1972). Most of the *P. aeruginosa* lectin activity is distributed inside the cell but is released after bacterial disruption, prompting the suggestion that *P. aeruginosa* lectins, released *in vivo* during bacterial disruption by local host defence mechanisms, may enhance binding of *P. aeruginosa* to host mucosa (Plotkowski *et al*, 1989). Further support for this theory of lectin mediated binding of *P. aeruginosa* in the lung is provided by the fact that the production of *P. aeruginosa* lectins *in vitro* is greatly stimulated by the addition of choline to the culture medium (Gilboa-Garber, 1972): phosphatidyl-choline, the major lung surfactant, can be degraded by elaboration of *P. aeruginosa* phospholipase C and phosphatase, yielding free choline (Plotkowski *et al*, 1989).

Adherence of *P. aeruginosa* to CF and non-CF mucins was confirmed in the microtitre assay employing Mab to measure the binding (Table 21). Although a propensity for adherence to CF mucin was not demonstrated, these results

confirm those of other investigators (Kubesch *et al*, 1988; Herrmann *et al*, 1989 and Ramphal *et al*, 1989), who also reported adhesion of *P. aeruginosa* to tracheobronchial mucins obtained from CF patients. Kubesch *et al* (1988) noted that a strain showed no preference for the mucin of its CF host and that there was no selection to specific oligosaccharide sequences of mucin: porcine submaxillary mucin was the best binding target for all *P. aeruginosa* strains tested. In this thesis, substantial binding of *P. aeruginosa* strains to porcine gastric mucin was observed in the microtitre assays and the electron microscopic studies. In the study of Ramphal *et al* (1989) differences in adhesion of *P. aeruginosa* to mucin glycopeptides from sputa of patients with CF and chronic bronchitis was observed, the authors suggesting that CF mucins may be altered after infection is established, resulting in less binding to some fragments. The above study also revealed that the most mucin-like glycopeptides from *P. aeruginosa*-infected CF sputa showed less bacterial adhesion than did the corresponding bronchitic or uninfected CF samples, and the most adhesive activity in CF samples came from a fraction containing O and N glycopeptides, which may be a degradation product of *P. aeruginosa* infection (Ramphal *et al*, 1989).

In the present study, adherence of *P. aeruginosa* to CF sol and gel mucins, fractions of CF gel mucin as well as sol and gel glycopeptide fragments was observed. Binding to the CF gel glycopeptides was particularly apparent indicating that the bacteria may have a special affinity for these glycopeptides. This may be due to more available binding sites (weight for weight) on the glycopeptides devoid of naked protein regions, or perhaps a result of better attachment of these fragments to the microtitre plate wells. Fraction D, the least acidic (less sialic acid) of the CF mucin fractions, showed greatest *P. aeruginosa* adhesion. Ramphal *et al* (1989) noted the absence of adhesion to glycopeptides containing a high level of sialic acid.

Adhesion to normal salivary mucins, especially the small salivary mucins (M_r 250,000) was observed, lending support to the hypothesis that the oral cavity may provide the site of initial colonisation. Komiyama *et al* (1987b) reported the sialic acid-induced aggregation of *P. aeruginosa* and speculated that such aggregation or adherence to sialic acid containing mucins in the oral cavities of CF patients may precede and facilitate colonisation of the lower respiratory tract. The study of Reddy *et al* (1985) revealed that there were no apparent qualitative differences in the neutral and sialic acid containing units in the oligosaccharide structures of low-molecular weight salivary mucins from a normal individual and one with CF. This would perhaps indicate that there is nothing in terms of the composition or structure of CF salivary mucins which confers increased susceptibility to *P. aeruginosa* infection. A carbohydrate analysis of purified mucins from sputa of CF and asthmatic patients showed no significant differences in the sugar components (Rose 1988).

In this study both CF and non-CF *P. aeruginosa* strains showed adherence to mucin, as demonstrated by both the microtitre assay and electron microscopy. In contrast, Herrmann *et al* (1989) reported that only *P. aeruginosa* strains isolated from patients with CF, displayed substantial affinity to mucin.

Given that the carbohydrate side chains on mucins are exposed and make up to 80% w/w of mucin (Rose, 1988), it would seem reasonable to speculate that sugar epitopes are the primary receptor for *P. aeruginosa* adhesion to mucin. Kubesch *et al* (1988) noted that the removal of oligosaccharide side chains abolished binding of bacteria to mucin, whilst Vishwanath & Ramphal (1985) showed that treatment of mucin with periodate (oxidises hydroxyl groups on carbohydrates), neuraminidase and heat inactivated influenza virus (binds to sialic acid) reduced *P. aeruginosa* adherence; pronase treatment had no effect. The study of Vishwanath & Ramphal (1985) concluded that NANA and GlcNAc may form the binding site for *P. aeruginosa* on human tracheobronchial mucin; similar observations were also made by Herrmann *et al* (1989), al-

though a broad heterogeneity of CF *P. aeruginosa* strains for their mucin carbohydrate receptor was reported. Sugar inhibition studies with the microtitre ELISA (Table 22) also reflected the possible participation of NANA and GlcNAc in the binding of *P. aeruginosa* to mucin. Sialic acid is also thought to be important in the cellular receptor for *P. aeruginosa* including buccal cells (McEachran & Irvin, 1985; Doig *et al*, 1989, Baker *et al*, 1990) and epithelial cells (Ramphal & Pyle, 1983b).

Interactions between bacteria and host mucins are thought to be important in bacterial colonisation of mucosal surfaces, eg binding of pathogenic *E. coli* to intestinal mucin may be protective for the host or may enhance colonisation and allow toxins to be delivered at high concentration to the underlying mucosa (Sajjan & Forstner, 1990); some oral bacteria bind to mucin molecules on teeth (Liljemark & Gibbons, 1972) and some *E. coli* strains bind to urinary tract mucus (Chick *et al*, 1981). In healthy individuals, tracheobronchial mucins serve to protect respiratory cells from bacterial colonisation by binding bacteria, the adherent or entangled bacteria being removed by mucociliary clearance (Reynolds, 1983; Reynolds, 1989). However, in the lungs of CF patients, mucociliary clearance is impaired (Fick, 1989) and binding of bacteria to the stagnant mucus would not be beneficial to the host. The particular affinity of *P. aeruginosa* for respiratory mucins, including specific adherence, could explain the propensity of the organism to colonise and persist in the static mucosal secretions of CF patients. Adherence of non-mucoid *P. aeruginosa* to mucin may act as a focus for infection, and subsequent development and establishment of alginate enclosed microcolonies, enmeshed in a mucin matrix. If the organisms colonise the mucosal blanket, adherent to mucins, then secretory immunity may be less effective since IgA itself may be mucinophilic (Ramphal, 1990). Binding of *P. aeruginosa* to mucin has also been shown to protect the organism from opsonophagocytic killing, but not poorly adhering strains including *E. coli*, *Staph. aureus* or group B *Streptococci* (Vishwanath *et al*, 1988). Thus *P. aeruginosa* could reside in the mucus and evade the

immunological response enabling it to coexist for long periods within the host. Although *H. influenzae*, *Staph. aureus* and *S. pneumoniae* also show some adhesion to mucus (Ramphal, 1990) the subsequent manifestation of the mucoid phenotype by *P. aeruginosa* is probably a key factor for its persistence and selection as the predominant coloniser in CF.

The structure of the terminal sugar chains of some mucin glycoproteins is the same as that determining the specificity of blood groups (Clamp *et al* 1978). The inability to secrete blood group substances may be an important factor in the development of chronic obstructive lung disease, blood group substances perhaps having a role in the interaction of microbes with mucosal surfaces (Haponik *et al*, 1985). However, a correlation between secretor status in CF patients and rate of correlation with *P. aeruginosa* has not been demonstrated (Haponik *et al*, 1985).

CHAPTER 2

ANTI-*P. AERUGINOSA* ANTIBODIES IN PATIENTS WITH CYSTIC FIBROSIS

2.1 ANTI-*P. AERUGINOSA* FLAGELLAR ANTIBODIES

In this study, analysis of flagella isolated from four non-mucoid *P. aeruginosa* strains confirmed the correlation between antigenic type and molecular weight (relative mobility) as described by Allison *et al* (1985). The appearance of the low molecular weight protein band in the gel profile of flagella isolated from JN61 (flagella type-a) appears to correspond to the observations made by Allison *et al* (1985). These authors noted a protein band of M_r 15,000-20,000 and reasoned that these small polypeptides were dissociated or protease nicked flagella, since increasing amounts of these bands and a decrease in native flagellin correlated with length of time in storage.

Bacterial flagella are known to be potent immunogens evoking both humoral and cellular immune responses (Newton *et al*, 1989). In this thesis anti-flagellar antibodies in serum, saliva and sputum from patients with CF were detected (Figures 24-26). The observation of similar levels of anti-flagellar type-a and type-b antibodies observed in many of the CF patients, especially those chronically colonised by *P. aeruginosa*, could result from infection with two different *P. aeruginosa* strains. The study of anti-flagellar antibodies in serum from CF patients reported by Anderson *et al* (1989) also indicated that colonisation with organisms of different flagellum type could have occurred at some point. Epidemiological evidence, however, suggests that CF patients remain colonised with a single strain of *P. aeruginosa* (Ogle *et al*, 1987) whilst other studies have reported that although a given serotype tends to persist in each patient, there is the occasional appearance of others (Diaz *et al*, 1970,

Horrevorts *et al*, 1990). Alternatively, the presence of the b or a₀ epitope in all seventeen flagellar serotypes (Ansorg, 1978) as well as a common N-terminal amino acid sequence in the flagellin protein (Rotering & Dorner, 1989) may account for some of the dual responses observed in the ELISA and immunoblot studies. Rotering & Dorner (1989) also observed that antibodies directed against type-a flagella can weakly bind to b-type flagellins and vice versa. Such cross-reactions were also observed in the immunoblot characterisations of the flagella preparations (Figure 23). Another possible explanation for the response to both flagellar types is diphasic variation in the flagellar antigens (Pitt, 1980) although no such variations were reported by Montie & Anderson (1988). In contrast, a pronounced antibody response to either type-a or -b flagella indicates either infection by *P. aeruginosa* of one flagellum type or perhaps by one single strain.

Shand *et al* (1988a) reported that many CF patients infected with *P. aeruginosa* show serum antibodies against flagellar preparations soon after the onset of colonisation; immunoblotting and ELISA studies showed an early response to flagellar antigen, which preceded the antibody response to other outer membrane components. In this thesis, the elevated anti-flagellar antibody levels, above the control range, observed in sera from some CF patients intermittently or non-colonised by *P. aeruginosa* adds support to the report of Shand *et al* (1988a). An early antibody response to flagella is likely to occur, since motility and expression of flagella are probably less important after initial colonisation, and establishment of chronic infection (Luzar *et al*, 1985).

Anti-flagellar IgA antibodies in saliva and sputum from patients with CF were also observed and may represent the local immune response to *P. aeruginosa* colonisation or infection. The raised anti-flagellar IgA antibodies in saliva or sputum from patients intermittently or even non-colonised by *P. aeruginosa* may indicate an early antibody response to recent colonisation by the organism. Although patients may be classified as non-colonised on the basis of sputum

bacteriology, colonisation by *P. aeruginosa* could be present, since sputum bacteriology does not necessarily reflect the current status of all lung regions (Przyklenk & Bauernfeind, 1988). Raised anti-flagellar IgA antibodies were particularly evident in patients chronically colonised by *P. aeruginosa*, although absence or reduced levels of antibodies in some of these patients may be caused by involvement in immune complex formation (Brown *et al*, 1989; Kronborg *et al*, 1989) or degradation by *P. aeruginosa* elastase (Heck *et al*, 1990).

ELISA screening of anti-*P. aeruginosa* flagellar IgA and IgG antibodies in patients with CF may provide a useful indication of the early onset of pulmonary colonisation by *P. aeruginosa* and provide a suitable parameter for instigation of anti-pseudomonal antibiotic therapy. Longitudinal monitoring of anti-flagellar antibodies in serum, sputum or saliva in patients classified as non-*P. aeruginosa* colonised may give a particularly early indication of *P. aeruginosa* colonisation.

The ability of anti-flagellar antibodies to inhibit motility, and indirectly chemotaxis, was shown by the inhibition of chemotaxis of two *P. aeruginosa* strains towards porcine mucin by the anti-flagellar type-b Mab (Figure 64). These studies reflect those of Montie *et al* (1982a) who demonstrated that antisera raised against flagellar preparations inhibited motility. Experiments with the burned mouse model involving vaccination with *P. aeruginosa* flagellar antigens (Holder *et al*, 1982; Montie *et al*, 1982a; Montie *et al*, 1987) or passive immunisation with anti-flagellar sera (Drake & Montie, 1987; Drake & Montie, 1988) have shown the protective efficacy of anti-flagellar antibodies in the inhibition of the lethal spread from a localised infection. Anti-flagellar antibodies may act by simple inhibition of the motility of *P. aeruginosa* (Montie *et al*, 1982a; Rotering & Dorner 1989) or by opsonophagocytosis of the organism (Anderson & Montie, 1989; Rotering & Dorner, 1989). Since chemotactically directed motility of *P. aeruginosa* may be important in initial lung colonisation of patients

with CF, vaccination with a bivalent vaccine consisting of type-a and -b flagellar antigens would appear a rational, but as yet unproven strategy for prevention of colonisation. The role of mucosal immunity and the protective capacity offered by secretory IgA antibodies, present predominantly in the upper and large airways, is not well understood. The main role of IgA antibodies appears to be inhibition of bacterial adherence to epithelial surfaces (Bienenstock, 1984) whilst IgG antibodies are required for optimal clearance of *P. aeruginosa* from the distal airway (Fick & Hata, 1989).

2.2 ANTI-*P. AERUGINOSA* LPS ANTIBODIES

a) Anti-LPS antibodies in CF sera

A number of studies have reported the presence of antibodies to *P. aeruginosa* LPS in CF patients (Moss & Lewiston, 1980; Pitt *et al*, 1985; Moss *et al*, 1986a; Jacobson *et al*, 1987; Cochrane *et al*, 1988b; Schaad *et al*, 1990). ELISA based methods for determining antibodies to *P. aeruginosa* have previously proved useful in monitoring both course and treatment of infection in CF (Brett *et al*, 1986a & b, Brett *et al*, 1987; Brett *et al*, 1990; Pedersen *et al*, 1987). The system developed by Brett *et al* (1986b) was based on whole cells representing commonly isolated O-serotypes whilst the ELISA method of Pedersen *et al* (1987) used a pooled sonicated extract of seventeen individual *P. aeruginosa* O-serotypes. In the present study, the ELISA system, based on PEV-02 (S-LPS) or core LPS from *P. aeruginosa* PAC605, was an attempt to refine existing techniques with a more defined antigenic preparation and monitor *P. aeruginosa* anti-LPS antibodies in CF patients. An ELISA system based on PEV-02, which consists of cell wall extracts of sixteen international O-serotypes of *P. aeruginosa* (IATS O:1-O:16) (Miler *et al*, 1977) or the common core component of LPS were considered appropriate coating antigens given that there are at least seventeen O-serotypes of *P. aeruginosa*. (Serotype O:17 is an extremely rare serotype and its omission from PEV-02 is not likely to have affected the results).

The ELISA data obtained (Figure 4) clearly shows an elevated anti-pseudomonal LPS antibody response in serum from CF patients chronically colonised by *P. aeruginosa* with low or undetectable levels of antibodies in intermittently and non-*P. aeruginosa* colonised patients. Elevated anti-pseudomonal antibodies in colonised patients and generally low levels of antibodies in intermittently colonised patients were reported by Brett *et al* (1986a & b), Pedersen *et al* (1987) and Brett *et al* (1990). In addition Fomsgaard *et al* (1988b) and Fomsgaard *et al* (1989) have reported that anti-LPS antibodies increased significantly at the onset of chronic infection and continued to increase to very high levels in the later stages of infection, whilst Moss *et al* (1986a) observed a thirty-fold elevation of *P. aeruginosa* LPS antibodies in twenty-one patients colonised with the organism compared to ten non-colonised CF patients and eleven healthy controls. In a more recent study Schaad *et al* (1990) also reported that antibody titres in chronically colonised patients were significantly higher than in patients with intermittent *P. aeruginosa* colonisation; CF patients without *P. aeruginosa* colonisation showed no detectable antibody titres to LPS. This latter finding corresponds to the results obtained in the present study ie absence of antibodies directed to S-LPS or core R-LPS in serum from non-*P. aeruginosa* colonised patients.

A response to core R-LPS is perhaps indicative of chronic colonisation by *P. aeruginosa* deficient in O-polysaccharide leaving the unsheathed common core LPS as the predominant LPS antigen. Such strains are typically polyagglutinable with O-typing sera (Hancock *et al*, 1983; Pitt *et al*, 1986) reflecting the presence of common antigenic structures in the core-lipid A region of the molecule (Fomsgaard *et al*, 1988a). The isolation of polyagglutinable and non-typable *P. aeruginosa* strains lacking high molecular O-antigen LPS, (as visualised by silver staining of polyacrylamide gels) from the patients chronically colonised by the organism reflects observations made by others. However, a strict relationship between non-typability and lack of O-antigen was not

observed, since some strains, including mucoid variants (eg JN10 in Figure 49), were non-typable yet shown to express LPS O-antigen. These observations were consistent with the findings of Cochrane *et al* (1988b).

The increased levels of IgG anti-LPS antibodies measured in patients previously non-colonised or intermittently colonised by *P. aeruginosa* indicate the presence and/or increasing persistence of *P. aeruginosa* in the respiratory tract (Figure 5). Brett *et al* (1986a) and Pedersen *et al* (1987) commented that the rate of increase in antibody titres to *P. aeruginosa* was fast or slow in different patients: some patients may show an increase in *P. aeruginosa* specific antibodies very shortly after *P. aeruginosa* organisms have been cultured for the first time, whilst in others colonisation with *P. aeruginosa* may persist for 1-2 years without eliciting a systemic immune response (Pedersen *et al*, 1987).

The urease ELISA, with either PEV-02 vaccine or core R-LPS, was found to be rapid, reproducible, suitable for routine use, and clearly distinguished between CF patients chronically colonised with *P. aeruginosa* and those non-colonised by the organism. The positive colour change of the urease ELISA substrate (yellow to purple) obtained with serum from patients chronically colonised with *P. aeruginosa* could easily be read visually, and could obviate the need for an automated plate reader. The ability to identify chronic *P. aeruginosa* colonisation of patients is useful, since implementation of anti-pseudomonal therapy as soon as chronic infection is diagnosed is known to improve patient prognosis (Szaff *et al*, 1983; Pedersen *et al*, 1987). Indeed, Fomsgaard *et al* (1989) recommend the quantitation of specific IgG and IgA antibodies to *P. aeruginosa* LPS by ELISA as a diagnostic tool for CF in order to distinguish between superficial colonisation and early or persistent infection caused by *P. aeruginosa*.

The absence of a systemic immune IgG response in the CF patients non-colonised by *P. aeruginosa* (Figure 4) may indeed indicate that these patients have

never been colonised by the organism. Alternatively, the absence of a systemic humoral response may be due to the presence of *P. aeruginosa* in sputum that was transient or non-pathogenic and failed to invade tissue and establish infection, or perhaps because there was an adequate local immune response utilizing secretory IgA antibodies. In the studies of Brett *et al* (1986b), Brett *et al* (1987) and Brett *et al* (1988) serum IgG antibody titres to *P. aeruginosa* surface antigens in patients with no known *P. aeruginosa* infection were monitored and often found to exceed the normal control range; in some cases the titre of IgG antibodies was greater than control values at or up to 24 months before the first isolation of *P. aeruginosa* (Brett *et al*, 1988). In a more recent study, Brett *et al* 1990 evaluated serum IgA antibodies in CF patients and reported that an increased serum IgA titre can give an even earlier indication than measurement of serum IgG titre, of the presence of *P. aeruginosa* in the respiratory tract of a proportion of patients. However, as the authors indicated, these results represent detection only of early *P. aeruginosa* tissue infection, although the organism may have been present for some time. This scenario is likely, given the fact that bacteriological examination of many of their patients was based on throat swabs, and bacteriological culture of sputum was only capable of detecting greater than 10^4 organisms/ml of sputum. Indeed, Przyklenk & Bauernfeind (1988) also found that 63% of their non-colonised CF group showed elevated antibody titres against some of the O-antigens, and conceded that the presence of *P. aeruginosa* may well have gone undetected in sputum, and furthermore, that sputum does not always reflect the current status of all lung regions. The bacteriology of sputum specimens in our laboratory is based on a quantitative culture procedure which allows detection of *P. aeruginosa* at concentrations as low as 10^2 CFU/ml sputum (Dr J.R.W. Govan, pers. comm.). In addition, although the difference between harmless colonisation and early infection is open to debate (Brett *et al*, 1988), isolation of *P. aeruginosa* even in low numbers should be regarded with caution since they ultimately form the reservoir from which mucoid variants arise: once established, eradication of the organism

even with aggressive treatment is extremely rare (Pier, 1985). From accumulated evidence it seems reasonable to suggest that detection of raised serum anti-*P. aeruginosa* antibodies, whether in the presence or absence of detectable organisms in sputum, should encourage instigation of anti-pseudomonal therapy in an attempt to improve patient prognosis.

In this study, *P. aeruginosa* LPS antibodies were not detectable in the sera obtained from ten blood donor controls (Figure 4). The absence of antibodies in these healthy individuals may be a reflection of the small sample size since anti-*P. aeruginosa* LPS antibodies in some normal individuals have been detected in certain studies (Fomsgaard *et al*, 1989; Grzybowski *et al*, 1989). Although *P. aeruginosa* is not usually found in the normal human microflora, it is ubiquitously distributed, and sometimes found in the intestinal tract and on normal skin. Thus multiple contacts of healthy subjects with *P. aeruginosa* may occur, trivial epidermal infections, or sporadically occurring colonisation of the gastrointestinal or genitourinary tracts, being possible sources of infection, and stimulation of anti-pseudomonal antibodies (Grzybowski *et al*, 1989). The low levels of anti-flagellar antibodies detected in some of the normal sera (Figure 24) may be indicative of such infections and illustrate the potent immunogenic nature of flagellar antigens.

The monovalent cell-wall extract components of the polyvalent *P. aeruginosa* vaccine PEV-02 are predominantly LPS (Miler *et al*, 1977). Serum anti-pseudomonal antibodies measured using PEV-02 as antigen, may represent (i) antibodies to any of the sixteen O-serotype antigens, (ii) antibodies directed against the common core region of the LPS or (iii) antibodies to the common A-band LPS of *P. aeruginosa* (Rivera & McGroarty, 1989) which is particularly expressed by non-typable clinical isolates commonly found in patients with CF (Lam *et al*, 1989). Other common LPS antigens have also been identified by Sawada *et al* (1985) and Kocharova *et al* (1988). In this study, serum from patients chronically colonised with *P. aeruginosa* produced a positive reaction

with most of the sixteen individual serotype vaccine components (Figure 6), again indicating the presence of antibodies directed to a common antigenic component of LPS and/or a number of serotype specific antibodies.

Examination of the immunoblots of serum from patients chronically colonised by *P. aeruginosa* revealed a response to the high molecular weight O-antigenic LPS of a number of the serotypes, as well as a strong response to the core-lipid A region of the molecule (Figures 16b & 17b). The response to different O-serotypes may indicate infection of the patient with a number of individual serotype strains or reflect a serologically cross-reactive component associated with high molecular weight LPS. As mentioned previously, patients tend to be colonised by a single strain with occasional, transient colonisation by others. The study of Diaz *et al* (1970) reported that seven out of seventeen patients showed a serum antibody response against two or more serogroups of *P. aeruginosa*, whilst immunoblot analysis of serum from a CF patient in the report of Schaad *et al* (1990) displayed bands in the O-side chain region of S-type LPS of three *P. aeruginosa* strains. Broadly reactive anti-*P. aeruginosa* antibodies in CF patients were also reported by Przyklenk & Bauernfeind (1988) who suggested a course of infections with several O-antigens or a more pronounced cross-reactivity among CF *P. aeruginosa* isolates than in non-CF isolates.

In contrast to the immunoblots obtained with serum from chronically colonised patients, sera from CF patients intermittently colonised with non-mucoid *P. aeruginosa* tended to show an antibody response primarily directed towards O-antigenic LPS of the typable, colonising strain as well as that of the corresponding standard serotype strain (Figures 11b, 12b, 13b & 14b). The immunoblots of sera from intermittently and chronically colonised patients suggests that antibodies directed to a number of serotype strains as well as core R-LPS, develop as infection progresses. Increased exposure of core antigen or other common LPS antigens, commensurate with loss of O-specific side chains,

coupled with persistent infection may account for these antibodies. The response to the core regions of a number of different serotype strains also indicates the presence of a common antigenic structure in the core-lipid A region of the molecule. Conversely a response to core LPS of some strains but not others, may indicate a certain degree of core heterogeneity.

The significant anti-core response found in sera from CF patients chronically colonised with *P. aeruginosa* is consistent with Hancock *et al*'s (1984) study in which 74% of CF sera reacted with the core portion of outer membrane preparations (presumed to represent core LPS). Furthermore Jacobson *et al* (1987) showed an elevated IgG antibody response to PAC605 LPS in *P. aeruginosa* colonised patients, where Western blotting demonstrated that sera from most of these patients displayed bands in the core R-LPS region.

The presence of a faint, anti-S-LPS response to PAC605 LPS blotted with some sera or sputa (eg Figure 16a) was unexpected since it is reported to lack high molecular weight smooth carbohydrate molecules (Rowe & Meadow, 1983; Meadow *et al*, 1984; Engels *et al*, 1985) and appeared to lack such molecules in silver stained SDS-polyacrylamide gels. However, the Western blot analysis of Meadow *et al* (1984) and Jacobson *et al* (1987) also demonstrated comparable faint, high molecular weight ladder bands. The disparity between the immunoblotting and silver staining may be caused by the relative insensitivity of the latter to detect small amounts of O-antigen LPS. The presence of small amounts of S-LPS could be a result of partial reversion to the parent strain or the presence of small amounts of S-LPS in native PAC605 cell walls.

b) Anti-LPS antibodies in CF sputum and saliva

The presence of anti-*P. aeruginosa* LPS antibodies in saliva and sputum from CF patients was also investigated in this thesis (Figures 8-10). The elevated levels of sputum IgA anti-LPS antibodies, particularly observed in chronically

colonised patients, is in accordance with the studies of Schiotz *et al* (1979) and Przyklenk & Bauernfeind (1988) who also reported significantly higher IgG antibodies in sputum from patients colonised with the organism. The immunofluorescent studies of Hann & Holsclaw (1976) also showed that *P. aeruginosa* cells isolated from CF sputum were coated with substantial amounts of IgA antibodies. The high levels of anti-core IgA antibodies in sputum from chronically colonised patients is indicative of persistent infection by *P. aeruginosa* expressing exposed core antigens caused by loss of smooth O-serotype antigen; the response to core LPS was confirmed by immunoblotting. As discussed for the serum antibody response to vaccine, the sputum IgA antibodies may be directed to any of the sixteen O-serotype antigens or directed to a common LPS component. The formation of immune complexes or degradation by *P. aeruginosa* elastase (Heck *et al*, 1990) may be responsible for an underestimation of IgA antibodies in sputum.

Detectable levels of anti-*P. aeruginosa* LPS antibodies (IgA) were also found in sputum from intermittently colonised patients as well as those classified as non-*P. aeruginosa* colonised. These antibodies may represent an early response to colonisation by *P. aeruginosa* or reflect previous transient colonisation by the organism. The demonstration of anti-LPS antibodies in sputum from the non-colonised patients is in contrast to the absence of detectable anti-LPS IgG in serum. The IgA response may represent antibodies produced locally by the bronchus associated lymphoid tissue, indicating the presence of the organism, at some point. The antibodies may prevent tissue invasion, and account for the absence of a systemic immune response.

In contrast to IgA, anti-LPS IgG antibodies were not detected in sputum from any of the CF patients. This observation concurs with the data of Cochrane *et al* (1988b) who noted only a very low IgG response in sputum from a *P. aeruginosa* colonised patient. Failure to detect LPS-specific IgG antibodies in sputum is consistent with the hypothesis that these antibodies combine with

LPS in the CF patients' lungs to form immune complexes (Høiby & Axelsen, 1973; Høiby *et al*, 1986; Moss *et al*, 1986a). In addition some of the IgG antibodies may have been degraded by *P. aeruginosa* elastase (Fick *et al*, 1985) or elastase from polymorphonuclear leucocytes (Doring *et al*, 1985). The inability to detect IgG antibodies may also be a reflection of its importance as an opsonizing antibody for clearance of antigen from the distal human airway (Fick & Hata, 1989; Reynolds, 1989).

Salivary anti-*P. aeruginosa* LPS IgA antibodies were also demonstrated in a number of CF patients including non-*P. aeruginosa* colonised patients (Figure 9). In immunologically sufficient individuals, secretory IgA is the major immunoglobulin species detected in salivary secretions and is considered to be the principal mediator of salivary immunity (Reynolds, 1983; Bienenstock, 1984; Kilian *et al*, 1988; Smith *et al*, 1989). The antibodies detected reflect a response to both S-LPS and core R-LPS antigens. As discussed for sputum, these antibodies in non-*P. aeruginosa* colonised patients may reflect an early response to the organism, which is not detectable by sputum bacteriology. Alternatively, they may indicate a number of previous transient encounters with the organism. In contrast to the immunological analysis of sputum, IgG anti-LPS antibodies were detected in salivary secretions from many of the CF patients included in the study (Figure 10). Although IgG is not the principal antibody of the upper respiratory tract it is known to be produced in significant amounts in saliva from minor salivary glands (Smith *et al*, 1989), and may be particularly evident during respiratory infection when transudation from serum may account for a large portion of the IgG seen. Some of the IgG detected in saliva may be from contaminating peripheral pulmonary secretions.

Longitudinal studies of anti-*P. aeruginosa* LPS antibodies in saliva and sputum from individual CF patients may prove useful in the assessment of patient prognosis. Przyklenk & Bauernfeind (1988) concluded that secretory IgA antibodies in sputum correlated well with the current clinical status of patients,

titres increasing during acute exacerbations and decreasing to normal values in more stable periods, adding that detection of serum anti-LPS antibodies was reflective of infections of a more chronic nature.

In summary, the presence of anti-*P. aeruginosa* antibodies in various anatomical sites in patients with CF is indicated by the data presented in this thesis. Although these antibodies, directed to O-antigenic and core LPS antigens as well as to flagella, may help protect CF patients from bacteremia and endotoxemia, it appears that they are unable to mediate elimination of the organism from the CF respiratory tract. The existence of antibodies at the stage of initial colonisation by non-mucoid *P. aeruginosa* does not appear to be beneficial: Brett *et al* (1990) reported serum IgA antibodies in a majority of their CF patients prior to isolation of the organism from the respiratory tract, but added that these were unable to prevent pseudomonal infection becoming established. Indeed, there is clear support for the hypothesis that a pronounced antibody response to *P. aeruginosa* antigens, once colonisation is established, has deleterious effects because of immune complex induced destruction of lung tissue (see Introduction). Vaccination of patients already harbouring *Pseudomonas* is contra-indicated because of the resultant immune complex mediated tissue damage (Langford & Hiller, 1984).

Given the poor pulmonary clearance characteristic of CF and the ability of *P. aeruginosa* to colonise the copious, static mucosal secretions, the protective value of antibodies induced prior to any pulmonary colonisation is uncertain. However, mucosal immunity has received relatively little attention (Pedersen *et al*, 1989b) and induction of a protective IgA response may well be the way to prevent colonisation. Strategies to improve pulmonary clearance via the mucociliary escalator, coupled with the presence of pre-existing mucosal antibodies induced by suitable immunisation, may, combined, present a more formidable barrier to initial colonisation. The nature of a successful, probably composite vaccine must consider the relevance and importance of the multitude of

P. aeruginosa virulence factors possibly involved in colonisation of the CF respiratory tract.

CHAPTER 3

MONOCLONAL ANTIBODIES AGAINST *P. AERUGINOSA* CORE LPS

3.1 CHARACTERISATION OF THE MONOCLONAL ANTIBODIES

Mabs recognising *P. aeruginosa* core LPS, unsubstituted by O-serotype antigens, were isolated and characterised. The Mabs were shown to be cross-reactive with all or most core LPS antigens belonging to a variety of standard and clinical *P. aeruginosa* strains. The pan-reactive, core specific response may support the idea of the presence of a common antigenic structure in the core region of *P. aeruginosa* LPS. The inner core region of *P. aeruginosa* LPS is believed to be homogeneous in *P. aeruginosa* and also common to most Gram-negative bacteria, whilst the outer core region is considered to be heterogeneous and different from that of other Gram-negative genera (Rowe & Meadow, 1983; Wilkinson, 1983; Kropinski *et al*, 1985). The concept of LPS core heterogeneity in *P. aeruginosa* is supported by the observations of Hancock *et al* (1983) and Yokota *et al* (1989). The former observed that core R-LPS specific Mabs interacted with only six of the seventeen serotypes and only two of sixteen CF isolates, whilst the latter, utilizing outer core specific Mabs, reported heterogeneity of the L-rhamnose residue on the outer core of *P. aeruginosa* LPS. The Mabs of Yokota *et al* (1989) bound to some, but not all of the Homma serotypes, binding beyond the O-serotype antigen, and binding particularly strongly to R-LPS strains.

In this study, immunoblot analysis of the Mabs showed that high molecular-weight polysaccharide prevented the Mabs from gaining access to the core epitope. However, the fact that the Mabs showed strong reactions with *P. aeruginosa* strains expressing S-LPS antigen, indicates that the covering of long O-polymer is unable to prevent access to unsheathed core antigen. This

may be a reflection of the low level of LPS on *P. aeruginosa* which contains a long O-polymer: Hancock *et al* (1983); Wilkinson (1983) and Rivera *et al* (1988) estimated the mole percentage of S-form LPS to be between 0.2 and 14%. In contrast, however, this low level of covering with O-polymer was able to inhibit the binding of the rough-core specific Mabs characterised by Sadoff *et al* (1985). The Mabs studied in this thesis were shown to react with various forms of *P. aeruginosa* LPS including extracted LPS preparations, cell wall extracts (PVA) and whole bacterial cells. In the case of some of the extracted LPS antigens with some of the Mabs, the ELISA OD readings tended to be weaker with extracted LPS compared to the other LPS forms: perhaps the core epitopes recognized by the Mabs are not optimally orientated in extracted LPS.

The detailed chemical structure of the LPS core region of the different mutant strains is shown in Figure 71. The gel profiles of the LPS defective core mutants (Figure 29) showing a leading band of core LPS followed by a higher molecular weight band (24-kilodaltons), correspond to LPS fractions containing a mixture of unsubstituted core, and core oligosaccharide with a single copy of side chain oligosaccharide attached (Wilkinson, 1983), and correspond to the core LPS profiles of other investigators (Meadow *et al*, 1984; Engels *et al* 1985; Jacobson *et al*, 1987). In a structural analysis of the core mutants, Rowe & Meadow (1983) found that PAC608 and PAC609 were able to produce high molecular weight fractions, whilst PAC611 was leaky and also produces some O-antigenic material. In the other defective mutants which lacked both O-antigenic side chains and different amounts of core material, there was an absence of core components containing a single copy of side chain oligosaccharide. The bands observed for these mutants (ie PAC605, PAC556 and PAC557) in this and the other studies may indicate the presence of leaky variants.

In order to give an indication of the residue(s) in the core region of *P. aeruginosa* which were epitopes for the Mabs, the binding to a series of LPS

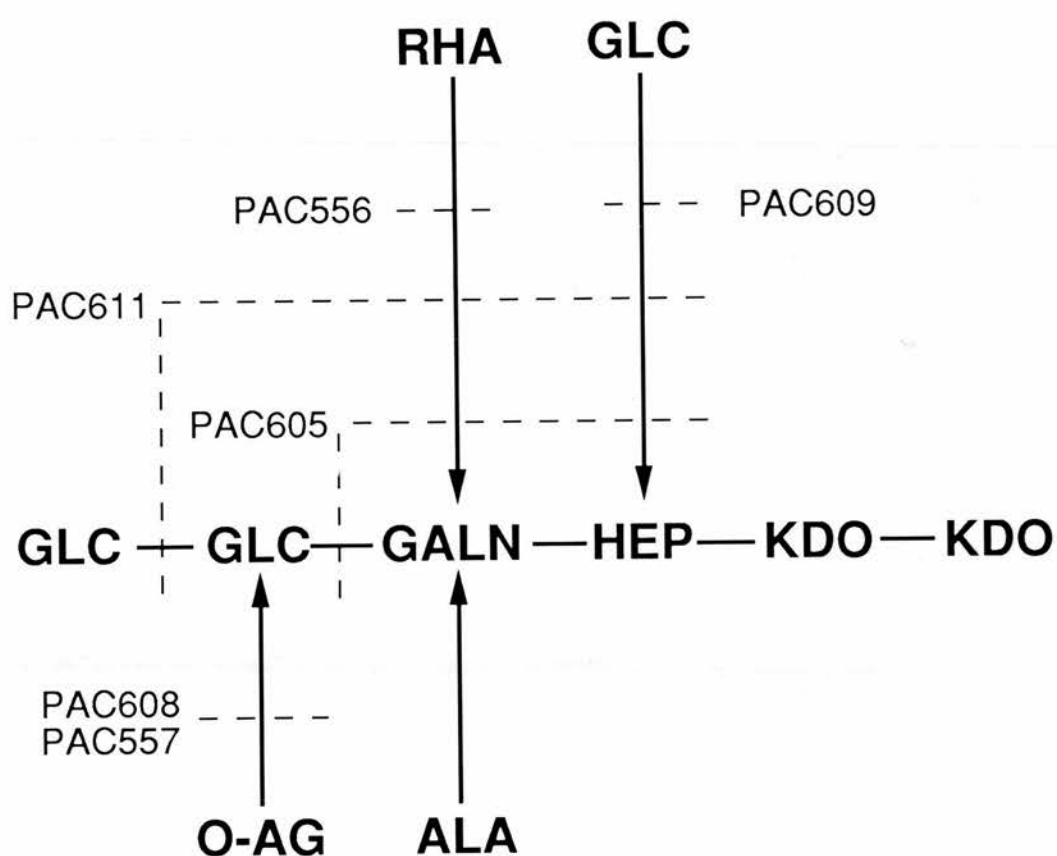


Figure 71. Detailed core LPS structure of *P. aeruginosa* PAC1R and its defective mutants (from Rowe & Meadow, 1983). ALA = alanine; GALN = galactosamine; GLC = glucose; HEP = heptose; KDO = *keto-deoxy octanoic acid*; O-AG = O-antigen; RHA = rhamnose.

defective mutants was studied. Analysis of the reactivities of the Mabs in ELISA (against whole cells and extracted LPS) and immunoblotting experiments gives a tentative indication of the Mab binding sites. Mabs 304.1.4 and 360.7 appear to show similar binding patterns: a reaction with all core mutants was observed although weak reactions against PAC605 LPS in ELISA and immunoblotting were noted. The binding sequence indicates that galactosamine and, to a lesser extent, its neighbouring glucose residue may form the epitope for Mabs 304.1.4 and 360.7. Discernment of the epitopic binding sites of the less avid Mabs 61.3.2, 73.5 and 334.4 is hindered by certain disparities in the ELISA and immunoblotting data. However, analysis of the ELISA whole cell results indicates that these Mabs also recognise an inner to mid core region, common to all the core mutants. The competition assays revealed that the binding sites of Mabs 304.1.4, 360.7 and 73.5 were probably slightly different, with Mab 61.3.2 having a similar binding site to Mab 304.1.4. A competitive ELISA involving the various core components may provide a more definitive answer for the determination of the epitopic site recognised by each of the Mabs.

Cross-reactivity of the Mabs with whole cells representing a number of other *Pseudomonas* spp implies that these species contain core LPS similar in composition and structure to *P. aeruginosa*. Alternatively their LPS may be structurally dissimilar but serologically cross-reactive with that of *P. aeruginosa*. The absence of a significant response to *P. maltophilia* confirms the observations of Neal & Wilkinson (1982) who reported similarities between the LPS of *P. maltophilia* and that of some *Xanthomonas* species. This analysis confirms their close taxonomic relationship based on rRNA/DNA hybridization (Palleroni, 1984), which is distinct from the other pseudomonads. The lack of a significant response to *P. cepacia* may also reflect some of the reported differences in the structure of *P. cepacia* LPS (Palleroni, 1984): the core polysaccharide is known to contain glucose, rhamnose and heptoses but no phosphorus.

Significant cross-reactions with *H. influenzae* were also absent. The LPS of *H. influenzae* is rough, the core oligosaccharide containing glucose, galactose, heptose and ketodeoxyoctonic acid with the variable presence of minimal amounts of glucosamine (Zamze & Moxon, 1987) and would appear to be different to that of *P. aeruginosa*. Although the inner core region of LPS from *P. aeruginosa* is believed to be similar to that of the enterobacteria (Wilkinson, 1983; Kropinski *et al*, 1985) no cross reactions of the Mabs with LPS and/or whole cells of *Salmonella*, *E. coli*, *Proteus*, or *Klebsiella* were observed. This would support the idea that the Mabs recognise epitopes in the outer core of *P. aeruginosa* LPS and which are absent from the core LPS of enterobacteria.

3.2 DEVELOPMENT OF A MONOCLONAL ANTIBODY ENZYME IMMUNOASSAY FOR THE DETECTION OF *P. AERUGINOSA* LPS

Pulmonary colonisation of patients with CF by *P. aeruginosa* can be diagnosed by bacteriological culture or, as described in this and many other studies, by the detection of *Pseudomonas* specific antibodies. However, in the early stages of asymptomatic colonisation such techniques may be limited by the absence of the organism in bacterial culture and the absence of anti-pseudomonal antibodies. The development of a Mab based enzyme immunoassay for the detection of the early emergence of *P. aeruginosa* antigens in saliva and sputum, facilitating early identification of respiratory colonisation, was considered a useful alternative to existing techniques.

The Mabs considered for the assay are core-LPS specific, pan-reactive with all *P. aeruginosa* strains regardless of serotype and therefore suitable for the detection of any of the twenty distinct serotypes (Liu & Wang, 1990) which may colonise individual CF patients. *P. aeruginosa* produces a number of intra- and extracellular antigens. Amongst these, LPS is specific, highly immunogenic and easily released into the surrounding medium (Cadieux *et al*, 1983; Kusama, 1983) and therefore considered an appropriate antigen for the detection of

P. aeruginosa in respiratory secretions from CF patients.

There has been increasing interest in the development of assays capable of detecting infectious agents directly in clinical specimens and which can be adapted to the rapid screening of a large number of specimens (Yolken, 1982, Yolken *et al*, 1983). Enzyme immunoassays have been developed for the detection of antigens of a variety of microorganisms eg *S. pneumoniae* and *H. influenzae* (Yolken *et al* 1983); meningococci (Sugasawara *et al*, 1984); *Bordetella pertussis* (Gustaffson & Askelof, 1988); *S. typhi* (Sadallah *et al*, 1989) as well as *P. aeruginosa* (Kusama, 1983). In the latter study, double antibody ELISA systems for the detection of the corresponding homologous LPS in solution were developed (Kusama, 1983).

Mab based systems have advantages over polyclonal antibody immunoassays: Mabs can be supplied in almost unlimited amounts once the hybridoma cell line is established; problems arising from batch variations are avoided when Mabs are used; Mabs generally show higher specificity since each molecule of antibody is directed at the antigen in question, and high concentrations of specific antibody can be obtained (Yolken, 1982; Harlow & Lane, 1988).

There are a number of ways in which enzyme immunoassay systems can be formulated to provide for the direct measurement of infectious agents in body fluids (Yolken, 1982). In this particular study a Mab based double antibody sandwich ELISA was developed for the detection of *P. aeruginosa* LPS in solution, and in saliva and sputum specimens from patients with CF. Bio-Mabs were used in the assays developed. Biotin labelling of antibodies is a simple, mild reaction giving high labelling efficiency and enables the antibodies to retain full biological activity. The stable monomeric conjugates generated have a number of advantages for use in enzyme immunoassays including the extraordinarily high affinity between biotin and avidin which provides stable complexes and has the potential for magnification at the level of cofactor-

enzyme interaction (Guesdon *et al*, 1979). Biotin-streptavidin reagents are also non-sticky in nature and therefore produce only low background reactions (Kendall *et al* 1983).

A biotin/Mab ratio of 1:1, 2:1 or 4:1 yielded usable reagents and corresponds to the results reported by others (Kendall *et al*, 1983; Yolken *et al*, 1983). Since one molecule of streptavidin can efficiently bind four molecules of biotin, multiple avidin-biotin interactions can be achieved, offering the possibility of increased sensitivity (Yolken, 1982). There are a number of ways of utilizing biotinylated antibodies. In this study the most efficient immunoassay system was one employing a complex of unlabelled avidin and alkaline-phosphatase labelled biotin to bind to the bio-Mab. Thus the ELISA system utilizing streptavidin-biotin-alkaline phosphatase complexes is more sensitive than the one utilizing bio-Mab reacted with streptavidin-directly labelled with enzyme, since in the case of the former each 'bridging' streptavidin molecule can bind three biotinylated alkaline phosphatase molecules to every biotinylated antibody.

The double Mab sandwich ELISA was shown to detect *P. aeruginosa* LPS in solution, with a detection limit of 0.1 ng/ml *P. aeruginosa* LPS or a minimum of between 10^3 - 10^4 cells /ml. A single Mab competitive inhibition ELISA was also attempted, but although the sensitivity was comparable to that of the sandwich ELISA, preliminary results were inconsistent with poor reproducibility. The principle of the competitive inhibition ELISA is that antigen (eg *P. aeruginosa* LPS in this case) is bound to the solid phase and a sample of test solution containing putative antigen is added together with labelled antibody (bio-MAb) specific for the antigen. Any antigen in the test solution will compete with the immobilised antigen for binding with the labelled Mab. The assay is quantified by the amount of labelled antibody bound to the solid phase. The advantages of this system are that it requires fewer incubation steps and requires only a single, labelled antibody compared to the sandwich ELISA technique (Harlow & Lane, 1988). However, problems may arise caused by

preferential binding of the labelled antibody to the solid phase compared to the antigen in solution. Antibody in clinical specimens may also interfere with the reaction by binding to the solid phase, or by preventing binding of labelled antibody to the antigen.

Sensitivity of a double antibody sandwich ELISA is dependent on a number of factors: i) ability of the capture antibody to bind to the plate, including the amount of immunoglobulin which can be tightly bound to a microtitre well (Yolken, 1982); ii) avidity of the capture antibody for antigen; iii) avidity and specific activity of the labelled antibody. This assay also requires two Mabs which have non-overlapping epitopes on the antigen showing non-competitive activity. In the sandwich ELISA system developed Mabs 304.1.4 and 360.7 were used. These Mabs showed strong cross-reactivity with *P. aeruginosa* LPS and appeared to be non-competitive. Mab 360.7 was used as the capture antibody since it showed the maximum avidity for *P. aeruginosa* LPS. Sensitivity of the assay may possibly be increased by further purification of the capture Mab (eg by ion exchange chromatography) since the preparations used still contained contaminating proteins as visualised by agarose gel electrophoresis.

The sandwich ELISA was able to detect various forms of LPS including extracted LPS, heat-killed whole cells and viable whole cells, and was therefore suitable for the detection of *P. aeruginosa* LPS in CF respiratory secretions, where it may exist in a variety of forms. Subsequent application of the system with saliva and sputum from CF patients indicated its ability to detect *P. aeruginosa* in respiratory secretions. Comparison of the results with the documented bacteriology for each of the patients showed a positive reaction for most of the patients known to be colonised by *P. aeruginosa*, although two false negatives were encountered: these may be caused by the precise nature of the LPS or by levels of LPS below the detection limit of the assay. Positive results were also noted for some patients considered to be non-*P. aeruginosa* colonised.

Longitudinal screening of such patients is required to confirm the significance of these results. However, it was of interest that the patient with the elevated positive absorbance reading for sputum, and previously considered to be non-*P. aeruginosa* colonised, has since had intermittent colonisation with non-mucoid *P. aeruginosa*. False positives caused by detection of *H. influenzae*, *P. maltophilia* and *P. cepacia* are unlikely given the fact that the Mabs failed to react significantly with whole cells from these organisms. Background reactions as well as false negative results were reduced considerably by boiling of the samples prior to use in the assay: heating liberates bacterial polysaccharides from antibody complexes (Dorskland & Berdal, 1980).

Further examination of this sandwich ELISA will indicate whether it is sufficiently sensitive and specific to provide useful information for the diagnosis and management of patients with CF: namely early identification of respiratory colonisation by *P. aeruginosa*, a basis for the determination of a patient's prognosis and design of rational therapeutic strategies, as well as a better understanding of the bacterial and host factors involved in early colonisation of the CF lung. Longitudinal studies including monitoring of *P. aeruginosa* LPS in CF respiratory secretions, coupled with measurement of *P. aeruginosa* specific antibodies and standard bacteriological examinations will be of value.

Given that ELISA systems tend to require more assay time than other immunoassay systems, eg immunofluorescence and latex agglutination (Yolken, 1982), the development of alternative immunoassays may be of benefit. Pfaller *et al* (1989) reported a direct fluorescent Mab test for the detection of *P. aeruginosa* in blood cultures, and Sofianou & Doumboyas (1989) used a coagglutination assay for the detection of soluble *P. aeruginosa* antigens in bronchial secretions. The feasibility of a latex agglutination test or a dot blot assay may also be considered. Commercial Mab based assay kits are currently available for detection of meningococci, pneumococci and *H. influenzae* in clinical specimens.

The immunoprotective potential of the Mabs and their possible use in passive immunotherapy (Holder, 1988) also remain to be evaluated. Use of the Mabs in patients with CF would probably be contraindicated because of the possibility of immune complex formation.

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